Recurrent hepatitis C virus infection post liver transplantation Predictors of fibrosis and treatment response.

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Recurrent hepatitis C virus infection post-liver transplantation:

Predictors of fibrosis and treatment response

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Abstract

Infection with chronic hepatitis C virus (HCV) is the leading indication for liver transplantation. Recurrence, however of HCV is universal after liver transplantation and subsequent fibrosis rates after transplantation are accelerated leading to significant morbidity and mortality. In addition, treatment of recurrent HCV after transplantation with pegylated-interferon (PEG) and ribavirin (RBV) are poorly tolerated and are associated with inferior treatment outcomes. The ability to therefore understand the mechanisms that relate to disease recurrence and therefore develop markers of fast fibrosis and treatment response would be beneficial for clinicians caring for these patients.

The role of donor and recipient rs12979860 IL-28B genotype was examined first. My results demonstrated recipient non-CC IL-28B genotype was associated with a rapid fibrosis (F≥2) progression at 12 months and increased risk of F≥4 post transplantation. The donor/recipient CC IL-28B combination was associated with attenuated fibrosis progression post transplantation.

Next, the role of CXCL10 in predicting fibrosis rates and treatment response was examined. My results that CXCL10 levels taken 6 months following liver transplantation correlated with fibrosis rates at 12 months and were predictive of rapid fibrosis progression at 12 months and F≥4. CXCL10 levels pre-treatment with PEG and RBV were predictive of successful treatment outcomes.

Finally, the role of microRNAs (miRNAs) were examined to delineate their role in predicting HCV recurrence. My results demonstrated intragraft expression of miRNA-146a, miRNA-19a, miRNA-20a and miRNA-let7e in patients with slow fibrosis progression (F<2) at 12 months. These miRNAs regulated the expression of cardinal genes implicated in promoting antifibrotic, antiangiogenic and anti-inflammatory pathways. miRNA-19a and miRNA-20a may also be possible serum biomarkers of fibrosis progression in patients with HCV post transplantation.

These experiments demonstrate and identify further predictors of fibrosis progression in patients with HCV recurrence after liver transplantation.

Declaration of Originality

'I, Deepak Joshi, declare that the work contained within this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis'.

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Acknowledgements

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Finally, I am indebted to all of the patients who I met and participated in this research. None of my work would be possible without you. Thank you.

Abbreviations

Ab	Antibody
ACR	Acute cellular rejection
ADAR	Adenosine deaminase acting on RNA
APC	Antigen presenting cells
ALD	Alcohol related liver disease
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APRI	Aspartate aminotransferase-to-platelet ratio index
AR	Acute rejection
AST	Aspartate transaminase
ATG	Anti-thymocyte globulin
AUROC	Area under the receiver operating characteristic curve
AVT	Anti-viral therapy
BMI	Body mass index
CCL	Chemokine ligand
CD	Cluster of differentiation
cDNA	Complimentary DNA
CI	Confidence interval
CIT	Cold ischaemia time
CLD	Chronic liver disease
CMV	Cytomegalovirus
CNI	Calcineurin inhibitor
CPA	Collagen proportionate area
CPS	Child Pugh score
CR	Chronic rejection
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DAAs	Directly acting anti-virals
DBD	Donation after brain death
DC	Dendritic cells
DCD	Donation after cardiac death
DDP4	Dipeptidyl peptidase 4
DM	Diabetes mellitus
DRI	Donor risk index
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EGFR	Epidermal growth factor receptor
EOTR	End of treatment response
ER	Endoplasmic reticulum
ESLD	End-stage liver disease
EVR	Early virologic response
F	Fibrosis
Fas-L	Fas Ligand
FCH	Fibrosing cholestatic hepatitis

G	Genotype
H&E	Henatoxylin and eosin
HA	Hyaluronic acid
HAT	Hepatic artery thrombosis
HBV	Hepatitis B virus
HBcAb	Hepatitis B core antibody
HCA	Hierarchical cluster analysis
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HOMA	Homeostasis model assessment
HPMT	High pure filter microtube
HR	Hazard ratio
HSC	Hepatic stellate cells
HVPG	Hepatic venous pressure gradient
ICAM	Intracellular adhesion molecule
ICU	Intensive care unit
IFN	Interferon
Ig	Immunoglobulin
IGD	Immune mediated graft dysfunction
IGF	Insulin-like growth factor
IL	Interleukin
INR	International normalised ratio
IP-10	Interferon gamma inducible protein-10
IR	Insulin resistance
IRF	Interferon regulatory factor 7
IRS	Insulin receptor substrate
ISG	Interferon sensitive genes
IVDU	Intra-venous drug users
JAK	Janus kinase
KIRS	Killer cell immunoglobulin-like receptors
LADR	Low accelerating dosing regimen
LDL	Low density lipoprotein
LPS	Lipopolysacharide
LS	Liver stiffness
LT	Liver transplantation
LTC	London transplant centre
MHC	Major histocompatibility complex
mRNA	Messenger RNA
miRNA	MicroRNA
MELD	Model for end stage liver disease
MF	Myofibroblasts
MIAME	Minimum information about a microarray experiment
MMF	Mycophenalate mofetil
MMP	Matrix metalloproteinases

MRE	Magnetic resonance elastrography
MSM	Men who have sex with men
NFκB	Nuclear factor kappa B
NHANES	National Health and Nutrition Examination Surveys
NI	Necro-inflammatory
NK	Natural killer
NKG2D	Natural killer group 2, member D
NO	Nitric oxide
NODAT	New onset diabetes after transplantation
NPV	Negative predictive value
NR	Non responder
NS	Non structural
OAS	Oligoadenylate synthetase
OR	Odds ratio
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PCH	Plasma cell hepatitis
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PDGF	Platelet derived growth factor
PEG	Pegylated interferon
PKR	Protein kinase R
PIIINP	Pro-peptide of type III pro-collagen
PNF	Primary non-function
PPV	Positive predictive value
qPCR	Quantitative PCR
rHCV	Recurrent HCV
RBV	Ribavirin
Rh	Rhesus
RNA	Ribonucleic acid
ROC	Receiver operator characteristic
ROS	Reactive-oxygen species
RPM	Revolutions per minute
RR	Responder relapser
RT	Reverse transcription
RVR	Rapid virologic response
S	Structural
SNP	Single nucleotide polymorphisms
SOF	Sofosbuvir
STAT	Signal transducers and activators of transcription
SVR	Sustained virologic response
TE	Transient elastography
TFLT	Time from liver transplantation
TGF-B1	Transforming growth factor – beta 1
TGFBR	Transforming growth factor receptor
TIMMPS	Tissue inhibitors of matrix metalloproteinases
TLR	Toll like receptor

TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor-related-apoptosis-inducing ligand
TRAIL-R	Tumour necrosis factor-related apoptosis-inducing ligand receptor
TRIF	TIR-domain-containing-adapter-inducing interferon-β
TW;	Treatment week
UNOS	United network for organ sharing
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
WIT	Warm ischaemia time

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Overview of Thesis

Chapter I - General Introduction to Hepatitis C Virus

A general introduction to HCV including epidemiology, structure and lifecycle. The pathogenic mechanisms of HCV infection including the immune response to HCV infection are also covered.

Chapter II - Introduction to the Present Study

The natural history of HCV recurrence post liver transplantation including recognised factors which influence fibrosis progression are reviewed. Treatment options including the new directly acting antiviral regimens are also discussed.

Chapter III – Methods

Comprehensive description of the various techniques used in the experiments performed in Chapters IV, V and VI.

Chapter IV – The role of donor and recipient IL-28B polymorphisms in HCV recurrence post liver transplantation

This study involved determining donor and recipient IL-28B genotype on stored patient samples. Their role was the examined in determining fibrosis rates in particular the development of F≥2 at 12 months and in predicting response to anti-viral treatment.

Chapter V – The role of CXCL10 in predicting HCV recurrence and treatment response post liver transplantation

This study involved quantifying CXCL10 levels at 6 months post transplantation and pre antiviral therapy to help determine fibrosis rates and response to anti-viral therapy post transplantation.

Chapter VI – Defining the role of microRNA in predicting HCV recurrence post liver transplantation and their role as biomarkers for HCV recurrence

This study involved determining the role of microRNAs in predicting HCV recurrence (fast (F≥2) versus slow (F<2) at 12 months) and identifying potential serum biomarkers.

Chapter VII – General Discussion

The thesis is concluded with a discussion of the main results from the three sets of experiments. The impact of my findings on the management of patients with HCV recurrence post transplantation are discussed including how the new directly acting antiviral agents will potentially change our approach.

Chapter I

General Introduction

1. Epidemiology

Hepatitis C virus (HCV) infection is a global epidemic and a leading cause of chronic liver disease (CLD) (Williams, 2006). Currently, HCV is the leading cause of death from liver disease and the leading indication for liver transplantation in the United States and United Kingdom (Kim, 2002). Data from the World Health Organisation (WHO) estimates that 3-4 million people are infected with HCV every year (WHO, 2012). Globally the prevalence of chronic HCV has been estimated to be between 2-3% (130-170 million people) (Lavanchy, 2009, Shepard et al., 2005)

Countries with high prevalence rates of chronic infection include Egypt (22%), Pakistan (5%) and China (3%) (WHO). Published data suggests that populations in Western Europe have a prevalence rate of antibody to HCV (anti-HCV) to be under 2.5% (Lavanchy, 2011). Estimating the prevalence of chronic HCV remains difficult and published figures are based solely on specific patient groups, not necessarily representative of the general population. These specific or high-risk groups include blood donors, intravenous drug users (IVDUs) and individuals with high-risk sexual behaviours. The National Health and Nutrition Examination Surveys (NHANES) have periodically collected and published data in the USA on HCV prevalence (Alter et al., 1999). Their most recent estimates suggest 1.6% or 4.1 million persons in the USA were anti-HCV positive, the majority of whom were born between 1945 and 1964 (Armstrong et al., 2006). Other high-risk groups include homeless persons and incarcerated persons (Chak et al., 2011).

In the Western World, the incidence of acute HCV infection particularly amongst HIV (human immunodeficiency virus) positive individuals has shown a recent increase, in particular amongst men who have sex with men (MSM) (Bollepalli et al., 2007, Fox et al., 2008, Low et al., 2008). Transfusion-acquired infection rates however, have been rare since the introduction of mandatory testing for all blood products/donors in 1991. IVDU related infection has also shown a decrease due to increased awareness and the combination of needle exchange programmes and safe injection education. Vertical transmission remains an important source especially given the global migration of individuals from countries with high prevalence rates i.e. Africa, Eastern Europe and South East Asia.

One patient group that are presenting increasingly with evidence of chronic HCV infection and its long-term complications are HIV co-infected individuals. One third of patients with HIV infection in Europe and the USA are co-infected with HCV, and up to 90% of deaths in HIV positive individuals with end-stage liver disease (ESLD) are attributed to HCV infection (Rosenthal et al., 2007). HIV/HCV co-infected individuals have a reduced rate of spontaneous HCV RNA clearance, with 80% of individuals going on to develop evidence of chronic infection (Graham et al., 2001).

2. Hepatitis C virus structure and lifecycle

HCV is a small (55-65nm), enveloped RNA virus (Figure 1.1a) which belongs to the *Flaviviridae* family, genus *Hepacivirus* and encodes a single polyprotein. Other members of the Flaviviridae family include yellow fever virus, dengue fever virus,

Japanese encephalitis virus and Tick-borne encephalitis virus. HCV is closely related to the human virus GB virus C (Lindenbach and Rice, 1999). HCV has at least 6 genotypes (1-6) with further subtypes (a, b, c, etc.). There is a high intra-genotype nucleotide sequence variability. The structure of the HCV genome is integral in the understanding of the new directly acting anti-viral (DAAs) agents and their action.

HCV is an enveloped virus that consists of a single positive RNA strand, that encodes a single, 9600 base pairs (Rosenberg, 2001). It posses both 5' and 3' untranslated regions (UTRs). A long open reading frame encodes the 10 viral proteins (Figure 1.1b). HCV virions exist as lipoviroparticles (LVP)

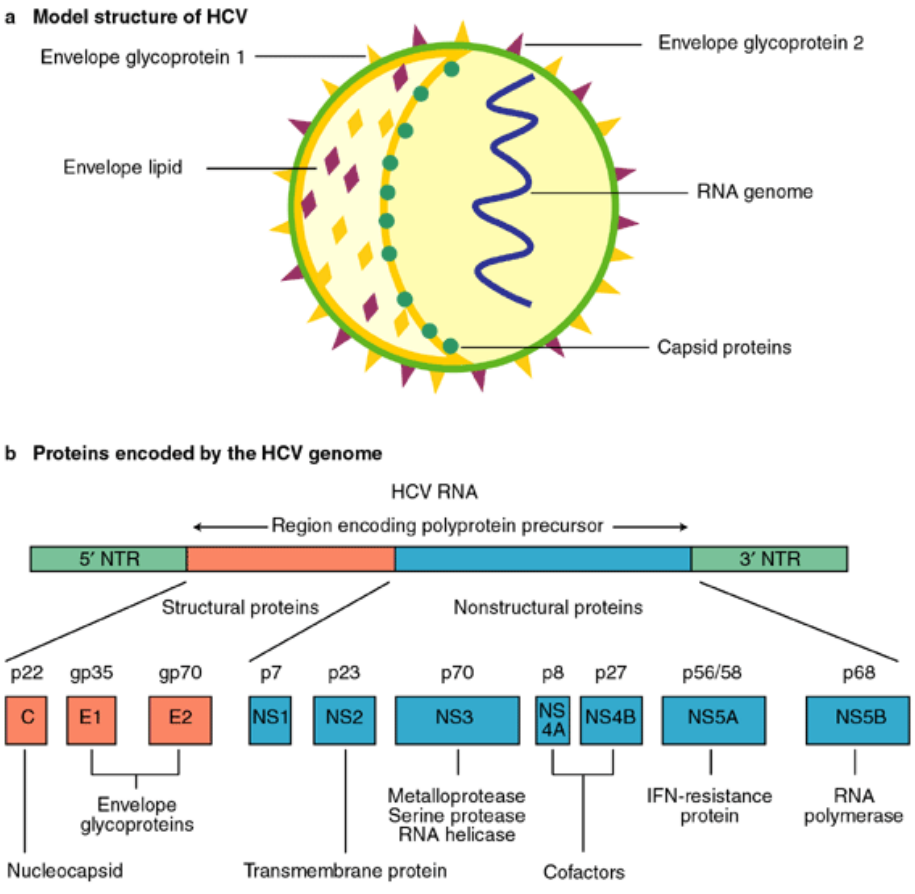


Figure 1.1 HCV structure and genome (Anzola and Burgos, 2003)

It consists of 3 structural (S) proteins (Core; E1; E2) in the N-terminal one-third and 7 non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) in the C-terminal two-third of the polyprotein. The core protein forms the viral nucleocapsid, and the envelope (E1, E2) proteins are glycosylated membrane proteins. NS-proteins have essential functions in viral replication. NS-proteins are released from the polyprotein following cleavage by HCV proteases (NS2-3 and NS3-4A) whilst S-proteins are released by host endoplasmic reticulum signal peptidases (Reed and Rice, 2000). The HCV genome consists of an open reading frame of approximately 9600 base pairs which is translated as a single polypeptide of approximately 3000 amino acids. It lacks a proof-reading function for the RNA-dependent RNA polymerase which accounts for the high mutation rate and genetic variability every 4-6 hours (quasispecies).

HCV replication largely occurs within hepatocytes. HCV can directly infect dendritic cells (DC), monocytes and lymphocytes (Pachiadakis et al., 2005, Muratori et al., 1996, Lerat et al., 1998, Bain et al., 2001, Sung et al., 2003). Infection of B cells is thought to drive clonal proliferation and account for cryoglobulinemia and related lymphoproliferative disorders that can occur with HCV infection (Rosa et al., 2005, Carbonari et al., 2005, Charles et al., 2008, Giordano et al., 2007). HCV binds to a receptor on the cell surface which leads to endocytosis of the viral particle. The LDL (low density lipoprotein) receptor and glycosaminoglycans facilitate initial binding followed E1-E2 interaction with CD-81 and scavenger receptor class B type I (SR-BI)(Barth et al., 2003, Agnello et al., 1999, Pileri et al., 1998). Claudin-1 (CLDN1) and occluding (OCLN) are necessary for entry (Ploss et al., 2009). The HCV genome is released into the cytosol following fusion of the viral envelope and the endosomal

membrane. HCV positive strand RNA is directly translated with production of HCV proteins. Expression of these HCV proteins induces intracellular membrane alterations (site of RNA replication). NS-proteins (NS3-NS5B) assemble and form a replication complex which is also responsible for RNA replication. A nucleocapsid is formed consisting of HCV RNA and S-proteins. These viral particles are then secreted.

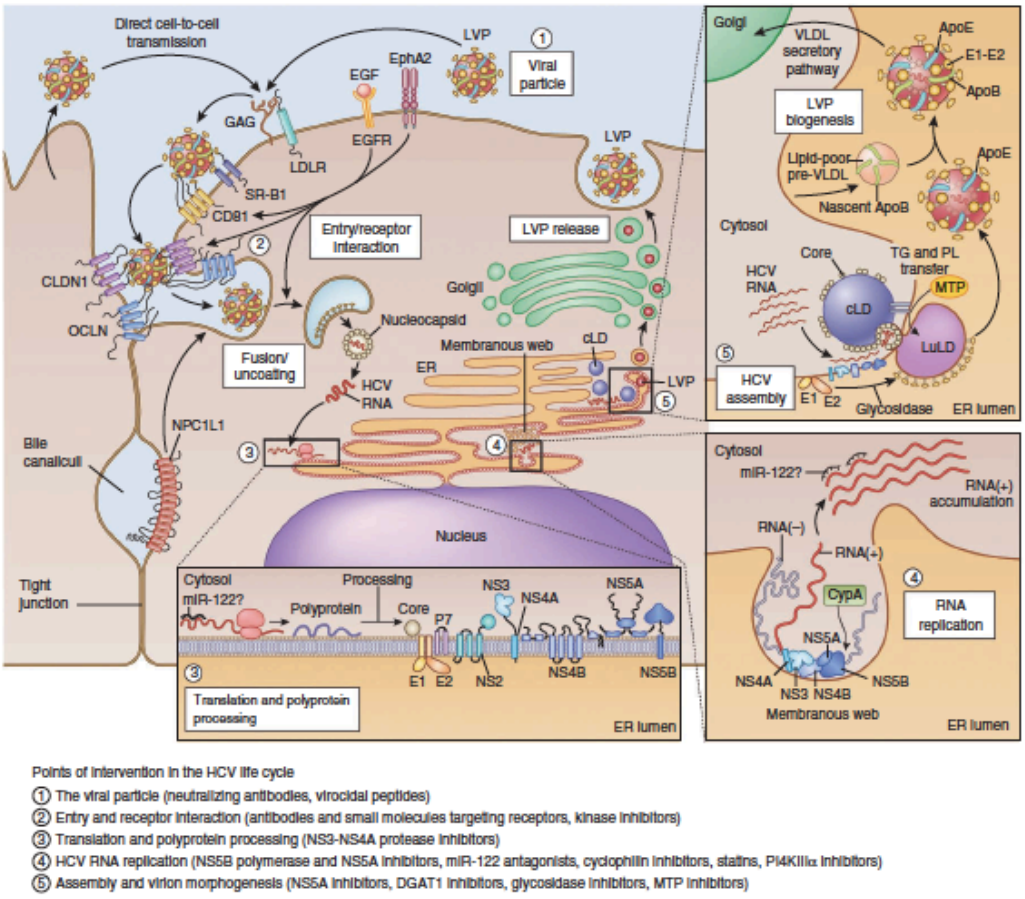


Figure 1.2. Life cycle of HCV replication (Scheel and Rice, 2013)
LVP, lipoviroparticles; miR, micro RNA; EphA2, ephrin receptor type A2; GAG, glycosaminoglycans; PL, phospholipids; TG, triglycerides;

2.1 Functions of HCV structural proteins

Core protein. The core protein is an RNA-binding protein that helps form the viral nucleocapsid. It also plays a role in cell signalling, apoptosis, carcinogenesis and lipid metabolism and steatosis.

E1 and E2 glycoproteins. These envelope proteins help create the viral envelope and also essential for virus entry and fusion as well as contributing to virus assembly (Bartosch and Cosset, 2006, Cocquerel et al., 2006).

2.2 Functions of HCV non-structural proteins

p7. The p7 polypeptide is a transmembrane protein and is located at the junction of the S- and NS- proteins. In vitro data suggests that p7 belongs to the viroporin family and acts as a calcium ion channel across the endoplasmic reticulum membrane (ER) (Gonzalez and Carrasco, 2003). It is not required for RNA replication but appears to be essential for viral assembly and the infectivity of HCV.

NS2. NS2 is a zinc dependent cysteine protease involved in the protease activity at the NS2/NS3 junction. It is not essential for the formation of the replication complex (Blight et al., 2000, Lohmann et al., 1999). The role of NS2 in its entirety remains unclear.

NS3 and NS4A. The NS3-NS4A protease is essential for the HCV lifecycle. NS3-NS4A protease activity is an essential component of the viral RNA replication complex and

also involved in attenuation of the host innate anti-viral response (Lindenbach and Rice, 2005, Foy et al., 2003). The first generation protease inhibitors, boceprevir acts on NS3 whilst Telaprevir inhibits the NS3-NS4A protease activity. The second generation protease inhibitor, simeprevir inhibits the NS3/4A protease activity. These drugs are predominately used in genotype 1 patients.

NS4B. NS4B is an integral membrane protein and acts as a membrane anchor for the replication complex.

NS5A. The NS5A is a membrane protein that's acts as a membrane anchor and is essential for genome replication (Appel et al., 2006, Seeger, 2005). Daclatasvir, ledipasvir and ombitasvir (formerly known as Abbvie, ABT-267) are NS5A inhibitors.

NS5B. NS5B is an RNA-dependent RNA polymerase that catalyses the replication of HCV (Behrens et al., 1996). It also interacts with cyclophilin B, stimulating RNA binding activity (Ishii et al., 2006). Cyclosporin A is known to inhibit cyclophilin B and therefore HCV replication in cell line culture (Watashi et al., 2003). Sofosbuvir is a nucleotide inhibitor that acts on the NS5B. The catalytic site of NS5B protein is highly conserved across all genotypes, making the nucleos(t)ide inhibitors that target this protein very appealing as a treatment option (Asselah and Marcellin, 2012). Dasabuvir (formely known as Abbvie, ABT-333) is also a NS5B RNA non-nucleoside polyemerase inhibitor that has been used in conjunction with ABT-450/ritonavir and ombitasvir (Andreone et al., 2014, Feld et al., 2014).

3. Pathogenesis of HCV infection

HCV can cause direct liver injury or indirect liver injury through immune mediated pathways. The typical tissue reaction in this setting is a chronic wound healing reaction which is characterised by the simultaneous presence of inflammation, tissue remodelling and regeneration. The deposition of fibrillar extracellular matrix (ECM) represents the best available solution aimed at maintaining tissue continuity in a context of extensive tissue necrosis/apoptosis. Fibrogenesis is a dynamic process. Indeed, newly deposited fibrillar ECM is rapidly degraded and tissue fibrosis is usually observed after a significant amount of time, when the rate of synthesis of fibrillar collagens (I, III, VI etc) by myofibroblasts exceeds the rate of degradation. This occurs for two main reasons: 1) the number of activated myofibroblasts reaches a peak hyperplasia partly in reason of a progressive resistance to apoptosis (activation phase), and 2) the perpetuation of the activation of this cell type is characterized by a progressive reduction of its ability to degrade and remodel fibrillar ECM (perpetuation phase).

In general, in chronic HCV infection as well other chronic liver diseases evolving towards cirrhosis, a significant accumulation of fibrillar ECM is observed only after a clinical course lasting several years and even decades. For example, in the large majority of patients with chronic HCV there is a long latency period (10-15 years) between HCV infection and the detection of minimal stages of fibrosis, in the presence of an evident and consistent degree of necro-inflammatory activity.

The end-point is however the same, progressive hepatic fibrosis, leading to cirrhosis. Liver fibrosis occurs due to the accumulation of extracellular matrix (ECM) components, a down-regulation of ECM-removing matrix metalloproteinases (MMPs) and an increase of tissue inhibitors of MMPs (TIMMPS) (Schulze-Krebs et al., 2005). In essence, a switch to a matrix secretion and accumulation phenotype occurs from a secretion and degradation phenotype (Henderson and Iredale, 2007). Interstitial collagen type I and III and type IV make up the majority of ECM components (Schulze-Krebs et al., 2005). Myofibroblasts (MF) produce these collagens, MMPs and TIMPs and are derived from either activated hepatic stellate cells (HSCs) or activated portal/perivascular fibroblasts (McCrudden and Iredale, 2000, Knittel et al., 1999, Friedman, 2000). HSCs reside in the space of Disse and have been identified as the main fibrogenic cell type in all forms of liver injury (Schuppan et al., 2003). HSCs store approximately 70% of the body's retinoid (Vitamin A) stores. Following liver injury, HSCs they differentiate into myofibroblast-like cells. HSCs become responsive to inflammatory cytokines and stimuli ('activation stage') before proliferating, migrating, contracting and producing the extra-cellular matrix (ECM) - 'perpetuation stage'

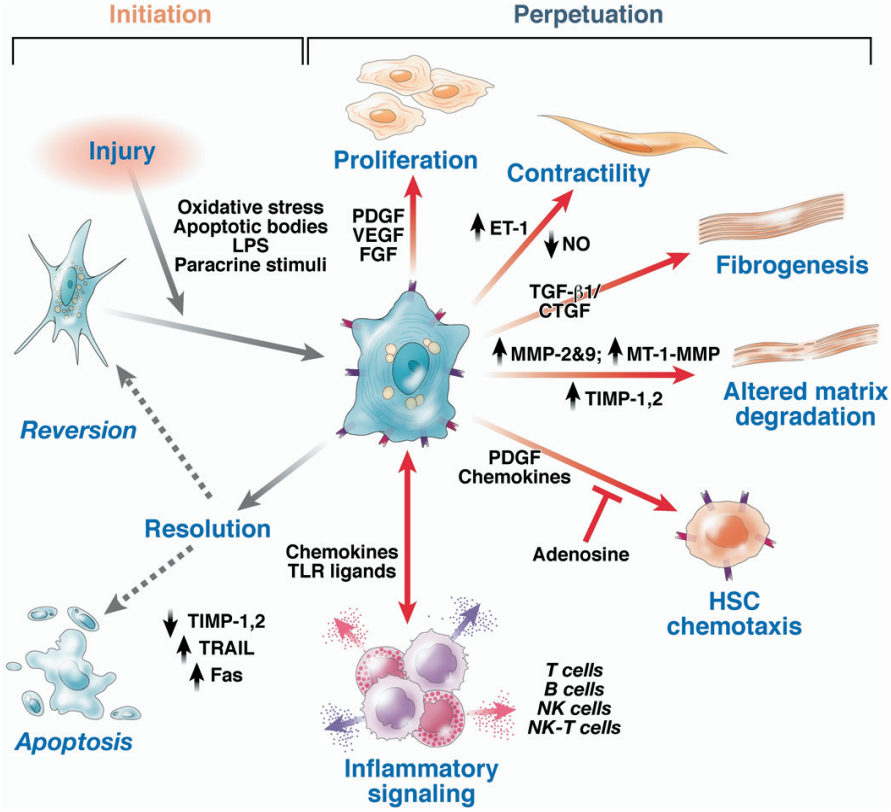


Figure 1.3 Pathway of hepatic stellate cell activation (Friedman, 2008a)

Although cirrhosis is the common result of progressive fibrogenesis, there are distinct patterns of fibrotic development, related to the underlying disorders causing the fibrosis. The typical pattern of chronic hepatitis C is defined “portal-central” and is the result of bridging necrosis progressing from around the portal tract towards the centrolubular vein. This leads to the preferential formation of portal-central fibrotic septa. In addition, this form of fibrogenic evolution is characterized by the presence of “interface” hepatitis and development of portal to portal septa and septa ending blind in the parenchyma, and by the rapid derangement of the vascular connections with

the portal system. This early derangement of the vascular architecture of the liver may lead to a significant degree of portal hypertension in the presence of a lower accumulation of fibrotic tissue when compared for example to chronic alcoholic hepatitis.

3.1 Necrosis

Necrosis secondary to oxidant stress can lead to HSC activation and is associated with an inflammatory cell infiltrate. This is considered to be a classical fibrogenic pathway.

3.2 Apoptosis

There is increasing data to suggest that HCV mediated liver damage is through the induction of apoptosis (Bantel and Schulze-Osthoff, 2003). These findings were made following the observation of acidophilic (Councilman) bodies in the liver lobule. During apoptosis, or programmed cell-death, cells become fragmented into small membrane-bound bodies (apoptotic bodies) and are then removed by phagocytosis (Henson et al., 2001, Savill, 2000, Thornberry, 1998). Apoptosis occurs through intrinsic and extrinsic pathways. Death receptors work via the extrinsic pathway and include Fas (CD95), tumour necrosis factor (TNF)-receptor-1 and tumour necrosis factor-related-apoptosis-inducing ligand receptors-1 and-2 (TRAIL-R1 and –R2) (Canbay et al., 2004). Death receptors are secreted in response to foreign antigens and then bind to their respective ligands, resulting in the production of caspases (Ashkenazi and Dixit, 1998). Intrinsic pathways involve disruption of the mitochondrial membrane resulting again in caspase activation. HCV infection results in the up-regulation of Fas in hepatocytes and

the induction of Fas-ligand (L) on T-lymphocytes which correlates with the severity of inflammation and results in apoptosis (Hayashi et al., 1997, Ferenbach et al., 1997, Pianko et al., 2001, Iio et al., 1998, Nelson et al., 1997, Erickson et al., 2001).

3.3 Angiogenesis

Angiogenesis in combination with inflammation contribute to the profibrogenic mileu. New blood vessel formation, sinusoidal modelling and HSC expansion have been demonstrated and are mediated by mediators such as VEGF (vascular endothelial growth factor) and PDGF (platelet derived growth factor) (Cassiman et al., 2002).

3.4 Transforming Growth Factor-Beta 1

TGF-B1 (transforming growth factor –Beta 1) is the major fibrogenic cytokine in the liver (Seki et al., 2007). It activates quiescent HSCs to smooth muscle alpha-actin expressing MF (Schulze-Krebs et al., 2005). TGFB expression is upregulated on hepatocytes following exposure to HCV non-structural proteins (Schulze-Krebs et al., 2005). TGF-B1 plasma levels and intrahepatic liver expression are elevated in chronic HCV infection, with higher levels associated with unsuccessful viral clearance (Flisiak et al., 2005). HCV can also lead to the production of reactive-oxygen species (ROS) which in turn can induce TGFB-1 (De Bleser et al., 1999, Garcia-Trevijano et al., 1999). Toll like receptor (TLR) 4 plays an important role in hepatic fibrogenesis by enhancing TGFB signalling (Seki et al., 2007).

Quiescent HSCs express CD81 and LDL-receptor, putative receptors for HCV suggesting that HSCs can be targeted directly by HCV (Bataller et al., 2004). Recombinant HCV proteins can also exert a direct fibrogenic effect on HSCs (Bataller et al., 2004). The continuous expression of HCV proteins on HSCs results in on-going production TGFB-1 (Bataller et al., 2004).

4. Immune response to HCV infection

4.1 Interferon pathways

Following infection with HCV, HCV RNA triggers the production of type-1 interferon (IFN) α/B within hepatocytes leading to the activation of IFN-sensitive genes (ISGs) via the JAK/STAT pathway (Thimme et al., 2006, Gale and Foy, 2005). Type-1 interferons are also produced by plasmacytoid dendritic cells (pDCs). ISGs include the OAS1/RNAse L system, RNA-specific ADAR1, P56 and PKR and are involved in degrading and destabilising viral RNA structures (Guo et al., 2004, Taylor et al., 2005, Hui et al., 2003, Pflugheber et al., 2002, Polyak et al., 2001a). IFN can also up-regulate the expression of HCV antigens on the surface of infected hepatocytes. However, HCV can dampen the IFN response through different mechanisms: increased expression of HCV core interferes with the JAK/STAT pathway of ISG induction by inhibiting STAT1 activation; increased expression HCV NS3/4A protein complex decreases IFN-β production; and HCV NS5A inhibits ISG expression through induction of IL-8 (Polyak et al., 2001a). Non-responders have higher ISG expression in pre-treatment liver biopsies compared to those who achieve a SVR or uninfected controls (Chen et al., 2005). Pre-activation of

ISGs appears to be confined to the liver and is not evident in PBMCs (Sarasin-Filipowicz et al., 2008). The NS3/4A protease in addition to processing viral proteins also plays an important role in the inhibition of type I IFN through the inactivation of TRIF and Cardif as well as interfering with NFκB (nuclear factor kappa B) production (Meylan and Tschopp, 2006).

The addition of ribavirin to IFN alters gene expression in pathways affecting HSC activation and maybe one possible explanation for the finding of reduced hepatic fibrosis in patients receiving long-term ribavirin monotherapy (Feld et al., 2007, Hoofnagle et al., 2003). A more recent study demonstrated the induction of several antiviral genes specifically by ribavirin in particular IRF7 and IRF9 (Thomas et al., 2011). IRF7 mRNA levels from PBMCs are known to correlate with the response to anti-viral therapy (AVT), whilst IRF9 has a direct antiviral action against HCV in tissue culture (Thomas et al., 2011, Taylor et al., 2007).

4.2 Natural killer cells

Natural killer (NK) cells are abundant in the liver making up approximately 50% of lymphocytes within the liver (Corado et al., 1997). Phenotypically NK cells are defined by the presence of the surface marker CD56 and the absence of CD3. CD56 ^{dim} NK cells represent the majority of NK cells (>90%) and express higher levels of killer cell immunoglobulin-like receptors (KIRs), CD16 and perforin and are therefore regarded to be more cytotoxic. CD56^{bright} NK cells constitute approximately 10% of NK cells and are deemed to be less mature and have the ability to differentiate into CD56^{dim} NK cells

(Chan et al., 2007).They also possess regulatory functions in the production of pro-inflammatory cytokines. $CD56^{bright}$ NK cells have less cytotoxic ability but express high levels of inhibitory receptors CD94:NKG2A. $CD56^{bright}$ NK cells exert their cytotoxic action through expression of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (Stegmann et al., 2010). In the absence of inflammation or infection, NK cells receive mainly inhibitory signals. NK cell activating receptors include NKG2D, CD94:NKG2C, CD94:NKG2E, NKp30, NKp44 and NKp46. Inhibitory cell receptors include CD94:NKG2A, 2DL1 and 2DL2/3.

NK cells do not require priming in order to either exert a cytoxic action or release cytokines. NK cells play an important role in the host defences against viral infections including cytomegalovirus (CMV) and influenza virus (Kubota et al., 1999, Mao et al., 2010). NK cells are activated by IL-12 and IFN-α/β and then secrete IFN-ɣ, tumour necrosis factor (TNF) and other chemokines (Biron et al., 1989). In HCV infection, the role of NK cells has been studied extensively. Binding of the HCV-E2 protein to CD81 results in the attenuation of NK cell function (Crotta et al., 2002, Tseng and Klimpel, 2002). NK cytotoxicity cell receptors are also downregulated by HCV via the HCV core protein (Herzer et al., 2003, Horner and Gale, 2009, Wen et al., 2008).

A seminal paper in 2004, reported a genetic polymorphism in the genes encoding the inhibitory NK cell receptor, KIR2DL3 and the human leukocyte antigen C group 1 (HLA-C1) ligand directly influenced resolution of HCV (Khakoo et al., 2004). Subsequent studies have described reduced number of NK cells peripherally and within the liver in patients with chronic HCV compared to healthy controls whilst one study showed no

difference (Deignan et al., 2002, Lin et al., 2004, Meier et al., 2005, Morishima et al., 2006, Par et al., 2002). A recent study demonstrated the induction of cytotoxic NK cell function in association with the first-phase virological response of IFN-alpha based therapy; NK cell TRAIL production peaked at 24 hour (Ahlenstiel et al., 2011). In the same study, patients that achieved an early virological response demonstrated an increased expression of NKG2D, NKp30 and CD16 (activating receptors) and decreased expression of NKG2C resulting in NK cell activation (Ahlenstiel et al., 2011). In contrast, another study demonstrated that patients with impaired viral kinetics have higher expression levels of inhibitory receptors (CD158a, CD158b and CD158e whilst patients who have an early viral decline have higher pre-treatment expression levels of the activating receptor NKp44 (Golden-Mason et al., 2011). On univariate analysis, pre-treatment levels of NKRp44, TRAIL and CD161 were associated with SVR whilst levels of the inhibitory receptors NKG2A, CD158e and CD158b negatively correlated with SVR (Golden-Mason et al., 2011). Successful antiviral therapy with pegylated-interferon (PEG) is associated with increased NK cell numbers and expression of activating NK cell receptor (NKG2D) and perforin (Dessouki et al., 2010).

4.3 Dendritic cells

Dendritic cells (DC) function as antigen presenting cells (APCs) and help link the innate and adaptive immune response. DCs can be divided into myeloid and plasmacytoid lineage. Myeloid DCs (mDCs) secrete IL-12 and TNFα and differentiate naïve CD4 cells towards a Th1 phenotype, whilst plasmacytoid DCs (pDCs) secrete IFN-α and drive naïve CD4 cells towards a Th2 phenotype (Liu, 2001, Liu et al., 2000). HCV core and NS3

proteins have been shown to impair DC function (Dolganiuc et al., 2003, Sarobe et al., 2002). DC and NK cells are also involved in reciprocal activation.

4.4 T lymphocytes

T-cells as part of the adaptive immune response are critical in the clearance of HCV (Lechner et al., 2000, Thimme et al., 2001). CD4+ T helper cells secrete antiviral cytokines and activate B cells and CD8+ T cells. Studies have shown that patients that develop HCV specific CD4+ T cells with subsequent IL-2 and IFN-ɣ production will go onto clear HCV compared to patients that generate a poor or weak CD4 T cell response (Kaplan et al., 2007, Urbani et al., 2006). CD8+ T cells become stunned and fail to proliferate in the setting of acute HCV function(Kasprowicz et al., 2008). However, CD8+ T cells dysfunction resolves once a CD4+ T cell response has been mounted (Lechner et al., 2000, Thimme et al., 2001, Urbani et al., 2006). Chronic HCV is related to loss of T cell function, with reduced HCV-specific CD4+ and CD8+ T cell responses.

Pre- liver transplantation, the development of chronic HCV infection appears to occur due to a combination of factors and in particular, exhaustion of the adaptive immune response (Spangenberg et al., 2005). In addition, the lack of proof reading capacity of the HCV RNA polymerase leads to HCV escape mutations and allows the virus further opportunities to escape from the individual's immune response. High levels of IL-10 are observed in chronic HCV infection and lead to inhibition of IFN-α, increased apoptosis of DCs and further dampens the T-cell response (Dolganiuc et al., 2006, Duramad et al., 2003).

Chapter II

Introduction to the Present Study

1. Natural history of HCV infection pre-liver transplantation

Acute HCV infection is invariably asymptomatic or characterised by non-specific symptoms i.e. lethargy, malaise, pyrexia. HCV RNA becomes initially detectable within 1-2 weeks of exposure before an increase in viral titres, peaking at approximately 8 weeks. The development of jaundice at the time of acute infection is associated with a decreased risk of chronic infection (Wiese et al., 2000). HCV anti-body (Ab) usually becomes detectable at the time of onset of symptoms but can remain negative in approximately 30% of patients (Farci et al., 1991).

Chronic HCV infection is defined by the presence of HCV RNA for at least 6 months after the onset of acute infection. Up to 85% of patients will go onto develop chronic HCV infection. Our understanding of the natural history of HCV infection has come from retrospective studies of iatrogenic induced HCV infection or population based cross sectional studies (Bellentani and Tiribelli, 2001, Kenny-Walsh, 1999, McMahon et al., 2010, Poynard et al., 1997, Vogt et al., 1999). Studies based upon women who received HCV contaminated anti-D immune globulin, used to prevent Rh (rhesus) immunisation during pregnancy, demonstrated that chronic infection occurred in approximately 55% over an extended period of time, usually decades (Kenny-Walsh, 1999). Individuals that are more likely to develop chronic HCV infection include those co-infected with HIV, of African descent and IVDUs (Alter et al., 1999, Graham et al., 2001, Thomas et al., 2000). No differences in rates of chronicity were demonstrated between men and women by two large studies (Alter et al., 1999, Bellentani and Tiribelli, 2001).

The natural history of chronic HCV infection is highly variable but is characterised by progressive fibrosis and in some the development of cirrhosis, liver failure and hepatocellular carcinoma (HCC). A recent meta-analysis using 111 studies, involving 33,121 individuals demonstrated that liver fibrosis progression was non-linear and disease progression was strongly influenced by duration of infection (Thein et al., 2008). The estimated prevalence of cirrhosis at 20 years and 30 years post infection was 16% and 41% respectively. The rate of fibrosis varies dramatically between different patients cohorts. Recognised risk factors that are associated with a more pro-fibrotic phenotype include male gender, race, age at infection, iron over load, cigarette smoking, cannabis use, duration of infection, alcohol consumption > 50g/day, hepatic steatosis in particular patients with genotype 3 HCV, increased BMI, diabetes and uncontrolled infections (HIV, HBV and shistosomiasis) co-infection (Adinolfi et al., 2001, Caronia et al., 1999, Freeman et al., 2001, Haydon et al., 1998, Mason et al., 1999, Poynard et al., 2001, Sachithanandan et al., 1997, Thein et al., 2008). HIV/HCV co-infected individuals have accelerated fibrosis rates compared to immuno-competent HCV mono-infected individuals, cirrhosis developing on average 12-16 years earlier (Bonnard et al., 2007, Soto et al., 1997, Joshi et al., 2011). Active HIV replication, low baseline CD4+ cell count and age > 25 years when HCV was acquired are specific risk factors for HIV/HCV co-infected individuals (Benhamou et al., 1999).

2. Natural history of HCV infection post-liver transplantation

Re-infection of the liver allograft is universal in individuals with HCV viraemia. During the anhepatic phase of transplantation, HCV RNA concentrations decrease (Fukumoto et al., 1996, Garcia-Retortillo et al., 2002). The rate of viral load decay does not appear to be related to the pre-transplant viral load or duration of the anhepatic phase but an association with the amount of blood loss during the anhepatic phase ($r=0.78$, $p<0.001$), the number of red blood cell concentrates transfused during the anhepatic phase ($r=0.71$, $p=0.001$) and during the entire surgical procedure ($r=0.76$,$p<0.001$) has been demonstrated (Garcia-Retortillo et al., 2002). The sharp fall in HCV-RNA occurs due to lack of virion production (Fukumoto et al., 1996). Following reperfusion of the graft, HCV-RNA titres continue to decrease secondary to large volume uptake of HCV by hepatocytes and the reticulo-endothelial system (Garcia-Retortillo et al., 2002). Within 12 hours of transplantation, HCV-RNA titres start to increase returning to pre-transplant levels within 4 days (Garcia-Retortillo et al., 2002). In some patients, HCV RNA titres can exceed pre-transplant levels (Feliu et al., 2004, Garcia-Retortillo et al., 2002). The development of more homogenous quasispecies population is one hypothesis postulated for this occurrence (Feliu et al., 2004). HCV-RNA titres increase thereafter and peak at the time of acute graft dysfunction which occurs between the first and 6^{th} month post-transplant (Gane et al., 1996a).

The clinical course of HCV post liver transplantation (LT) is that of progressive and accelerated fibrosis resulting in cirrhosis, graft loss and possible re-consideration for LT (Berenguer, 2002, Charlton et al., 1998, Gayowski et al., 1997). Histological evidence of

HCV has been demonstrated as early as the second week following LT (Garcia-Retortillo et al., 2002, Saraf et al., 2007). Activation of HSCs is clearly an important event. Studies using α-SMA (alpha smooth muscle actin) staining have shown that HSCs are activated as early as 4 months post transplant resulting in severe fibrosis (Cisneros et al., 2007, Gawrieh et al., 2005). This early involvement of HSC reflects a common feature of post-LT HCV-induced liver fibrosis often characterised by a pattern of evolution, i.e. periportal fibrosis associated with significant capillarisation of sinusoids, different from what normally observed in pre-transplant chronic HCV infection. This potentially leads to misclassification when staging fibrosis with METAVIR in the post-LT setting since this semi-quantitative scoring system does not consider sinusoidal fibrosis. Accordingly, a more appropriate assessment in this setting could be obtained by employing quantitative measures of fibrosis such as the morphometric evaluation of the collagen proportionate area (Bedossa et al., 2003, Standish et al., 2006).

Approximately 5% of patients will develop a severe cholestatic recurrence of HCV, which is characterised by rapid portal fibrosis, hyperbilirubinaemia, cholestasis and liver failure(Davies et al., 1991, Zylberberg et al., 1997). Histological features of recurrent HCV post liver-transplantation demonstrate many features seen in patients with HCV pre-transplantation. They include hepatocyte ballooning, intrahepatic cholestasis and biliary ductular proliferation without lobular inflammation. Early changes (0-2 weeks) include mild lobular inflammation and cell swelling with scattered apoptotic bodies (Figure 2.2).

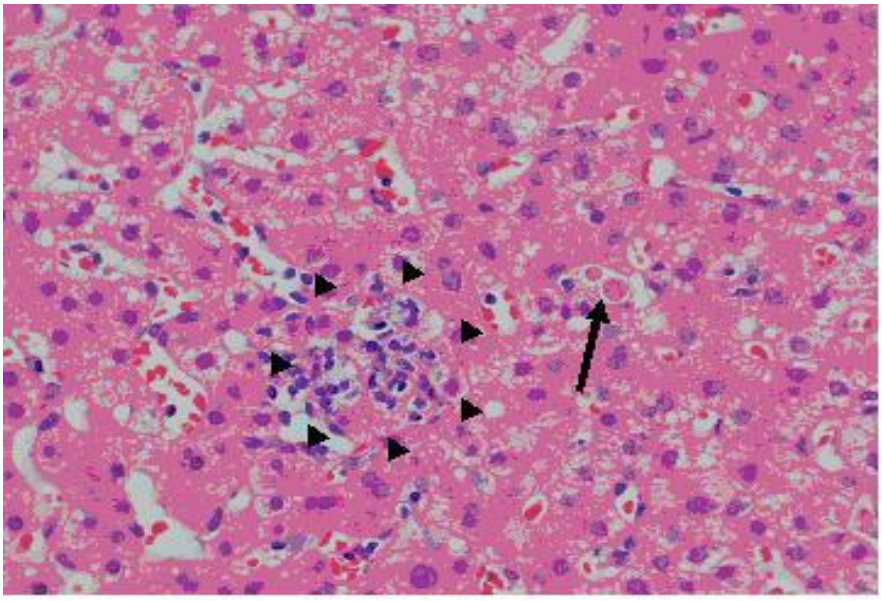


Figure 2.1 Early histopathological changes of HCV recurrence post liver transplantation.
H&E 400x. Mild lobular inflammation (arrow heads) and two acidophilic bodies (arrow). Provided by Dr Alberto Quaglia.

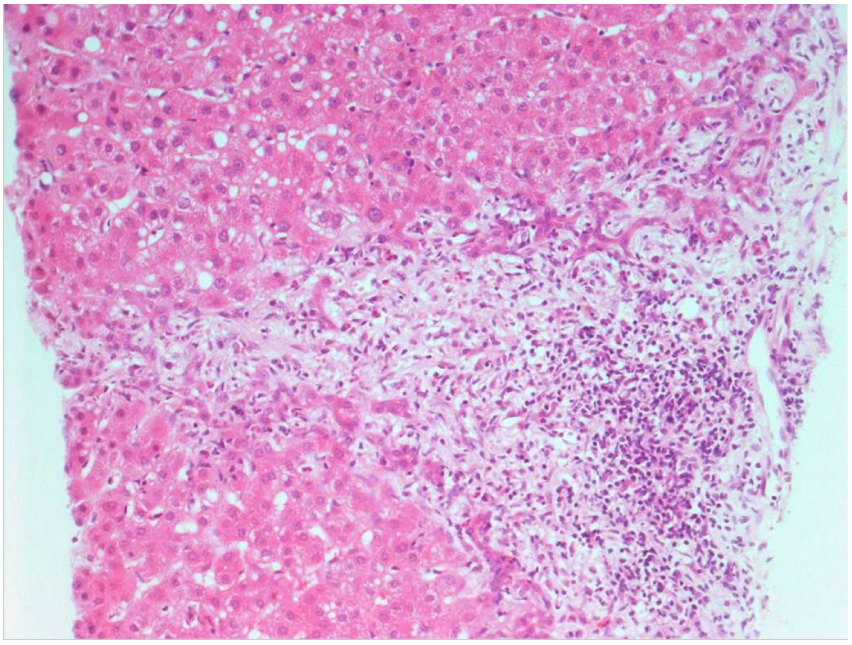


Figure 2.2 Fibrosing cholestatic hepatitis. H&E. Image demonstrates a portal lymphoid aggregate and ductular reaction at the limiting plate in a cholangiolitic pattern. Provided by Dr Alberto Quaglia.

At one year, nearly 50% of individuals will demonstrate significant histological evidence of HCV recurrence (Figure 2.3), 44% being cirrhotic within 5-10 years (Feray et al., 1992, Gane et al., 1996b, Prieto et al., 1999, Rosen et al., 1996, Rosen et al., 1998). Studies that have used serial protocol liver biopsies have demonstrated a more rapid fibrosis progression rate/year compared to immunocompetent patients pre-LT (Baiocchi et al., 2008, Berenguer et al., 2000, Belli et al., 2007, Firpi et al., 2004, Gane et al., 1996a, Neumann et al., 2004a, Pelletier et al., 2000, Sebagh et al., 2003, Wali et al., 2002, Yilmaz et al., 2007). Fibrosis stage $\geq$ 2 and severity of necro-inflammatory activity at 12 months appear to be important predictors of cirrhosis at 5 years (Firpi et al., 2004, Neumann et al., 2004b, Prieto et al., 1999). The clinical impact of this accelerated disease course is that greater than 70% of patients will have an episode of hepatic decompensation by 3 years therefore necessitating consideration for re-transplantation (Berenguer et al., 2000, Pruthi et al., 2001). Recurrent HCV remains a leading cause of morbidity and mortality post-LT.

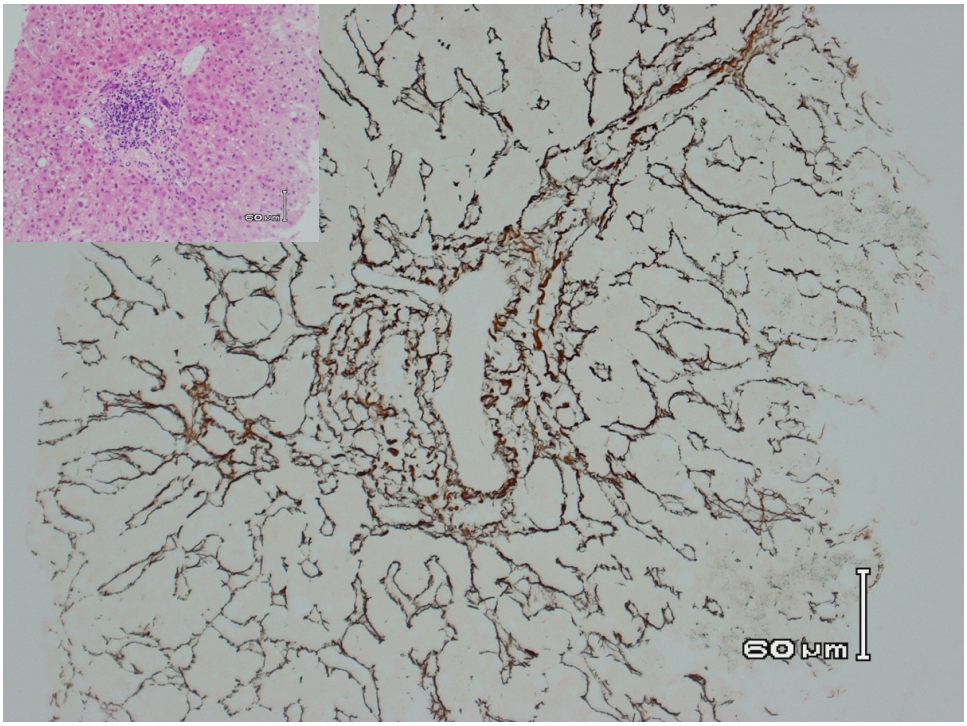


Figure 2.3 Liver biopsy 12 months post transplantation demonstrating HCV recurrence (F<2). H&E stain inset demonstrating a lymphocytic portal infiltrate. Reticulin stain (larger image) demonstrating portal expansion. Provided by Dr Alberto Quaglia.

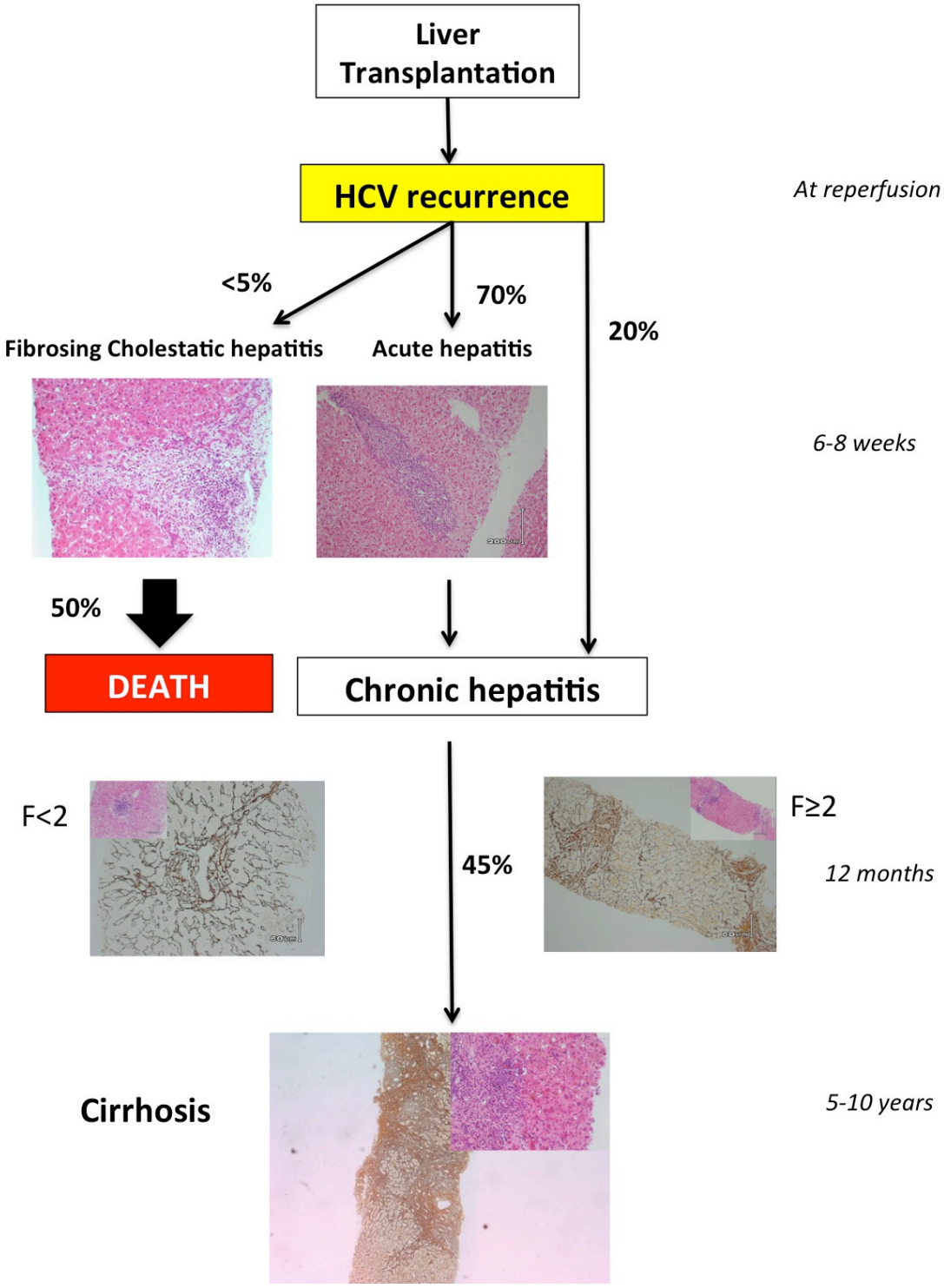


Figure 2.4, Natural history of HCV infection post liver transplantation Adapted from (Joshi et al., 2014)

3. Patient and graft survival

Initial single centre studies reported similar outcomes between HCV positive and HCV negative patients (Boker et al., 1997, Ghobrial et al., 2001, Sanchez-Fueyo et al., 2002). One of the first reports intimating inferior long-term outcomes of HCV infection following liver-transplantation was a single centre, UK study (Gane et al., 1996b). Although the data demonstrated comparable patient and graft survival at both 1 and 5 years between HCV-positive and HCV-negative patients (79% and 70% vs. 75% and 69%, p=NS), there was a suggestion that with longer follow-up, HCV-positive patients may have poorer outcomes (Gane et al., 1996b).

Since then, large transplant database studies have confirmed this initial observation, reporting significantly inferior graft and patient survival rates in patients with HCV (Forman et al., 2002, Mutimer et al., 2006, Thuluvath et al., 2007, Zekry et al., 2003). A European based study also demonstrated that whilst 1-year graft survival rates in HCV-negative patients had improved slowly over a 10-year period (1991-2000), the opposite was evident in HCV-positive patients (Berenguer et al., 2002, Ghobrial et al., 2001). One subsequent single centre study, reported long-term outcomes post-LT over a period of 20 years and clearly demonstrated inferior survival rates in patients with HCV (81% at 1-year, 68% at 5-years, 62% at 10-years) compared to other aetiologies (Busuttil et al., 2005).

Author	N (HCV/non-HCV)	Outcomes
(Gane EJ et al., 1996b)	149/623	*Inferior outcomes in HCV:* 1 & 5-year patient survival HCV: 79% & 70% 1 & 5-year patient survival non-HCV: 75% & 69%
(Berenguer M et al., 2002)	283/239	*Inferior outcomes in HCV:* 1 and 5-year patient survival HCV:77% & 61% 1 and 5-year patient survival non-HCV:87% & 76%
(Thuluvath et al PJ.,2007)	7,459/20,734	*Inferior outcomes in HCV:* 3-year patient survival rate HCV: 79% 3-year patient survival rate non-HCV: 81% Improved graft and patient survival rates in non-HCV patients. No change in HCV patients
(Mutimer DJ et al., 2006)	4,736/5,406	*Inferior outcomes in HCV:* Improved 10-year graft and patient survival in non-HCV patients compared to HCV ($p<0.001$)
(Sanchez-Fueyo A et al., 2002)	122/215	*No difference in outcomes between HCV and non-HCV:* 3,5,& 7-year graft survival HCV: 86%, 78% &71% 3,5,& 7-year graft survival non-HCV:85%,79% & 74% 3,5,& 7-year patient survival HCV: 89%,82%& 74% 3,5,&7-year patient survival non-HCV: 89%,84% and 79%
(Boker KH et al., 1997)	61/474	No difference in outcomes between HCV and non-HCV: 2,5& 10-year survival HCV: 67%,62% and 62% 2,5 & 10-year survival non-HCV: 62%, 57% & 52%

Table 2.1 Outcomes for transplantation in HCV-positive patients in comparison to HCV-negative patients.

4. Re-transplantation for HCV

Indications for re-transplantation include primary non-function (PNF), hepatic artery thrombosis (HAT), chronic rejection and disease recurrence. In general, reported outcomes for re-transplantation irrespective of the underlying aetiology are associated with a poorer clinical outcome (Ghabril et al., 2007). Re-transplantation for recurrent HCV post-LT however, remains a controversial issue. Re-transplantation outcomes for emergency indications i.e. PNF or HAT in HCV-positive individuals are comparable to HCV-negative individuals. The occurrence of a severe fibrosing cholestatic recurrence of HCV pre the DAA era was regarded as an absolute contraindication to re-transplantation.

Initial reports suggested poorer outcomes after re-transplantation in HCV-positive individuals but more recent studies have demonstrated no differences in patient or graft outcomes in HCV – positive individuals (Ghabril et al., 2008, McCashland et al., 2007). Analysis of the UNOS database has demonstrated improved graft survival rates following re-transplantation post 2002 in all patients irrespective of aetiology including HCV positive individuals (Ghabril et al., 2008). This observation would certainly support the hypothesis of 'a learning curve' by transplant centres in the identification and management of HCV re-transplant recipients. Predictors of an increased risk of mortality post re-transplantation in HCV-positive individuals include MELD score >25, increased donor and recipient age, elevated serum bilirubin, elevated INR and elevated creatinine. Careful evaluation and selection is therefore advocated of patients with recurrent HCV for re-transplantation.

Author	N	Outcomes	Predictors
(Bahra et al., 2007)	18	5-year patient survival 59%	-MELD > 25 -Bilirubin
(Carmiel-Haggai et al., 2005)	47	31 (66%) died post re-LT	-Donor age > 60 -Clinical HCV recurrence -Cirrhosis
(Watt et al., 2003)	899	1-year patient survival: 61% 3-year patient survival: 50% 5-year patient survival: 45%	MELD > 25
(Ghabril M et al., 2008)	1034	1-year patient survival: 70% 1-year graft survival: 63% 3-year patient survival: 59% 3-year graft survival: 53%	-Recipient age -MELD>25 -Donor age>60 -WIT≥75mins - Re-transplant interval < 1 year
(McCashland T et al., 2007)	43	1-year patient survival: 69% 3-year patient survival: 49%	-
(Roayaie et al., 2003)	42	-	-Prothrombin time -Donor age
(Pelletier et al., 2005)	464	Mortality risk for HCV: HR 1.3, 1.1-1.54, p<0.01	-Recipient age -Creatinine -Donor age -Admission to ICU
(Rowe et al., 2010)	34	5-year graft survival 45%	-
(Andres et al., 2012)	1422	-	-Recipient age at first transplant -Interval between transplants -Donor age -Creatinine* -INR* -Albumin* * at second transplant

Table 2.2 Studies evaluating the role of re-transplantation in patients with recurrent HCV (excluding patients with primary non-function).

5. Factors influencing HCV recurrence post liver transplantation

HCV recurrence is influenced by a complex combination of recipient, donor, immunosuppresive, viral, infectious and surgical factors (Figure 2.5), some of which potentiate each other.

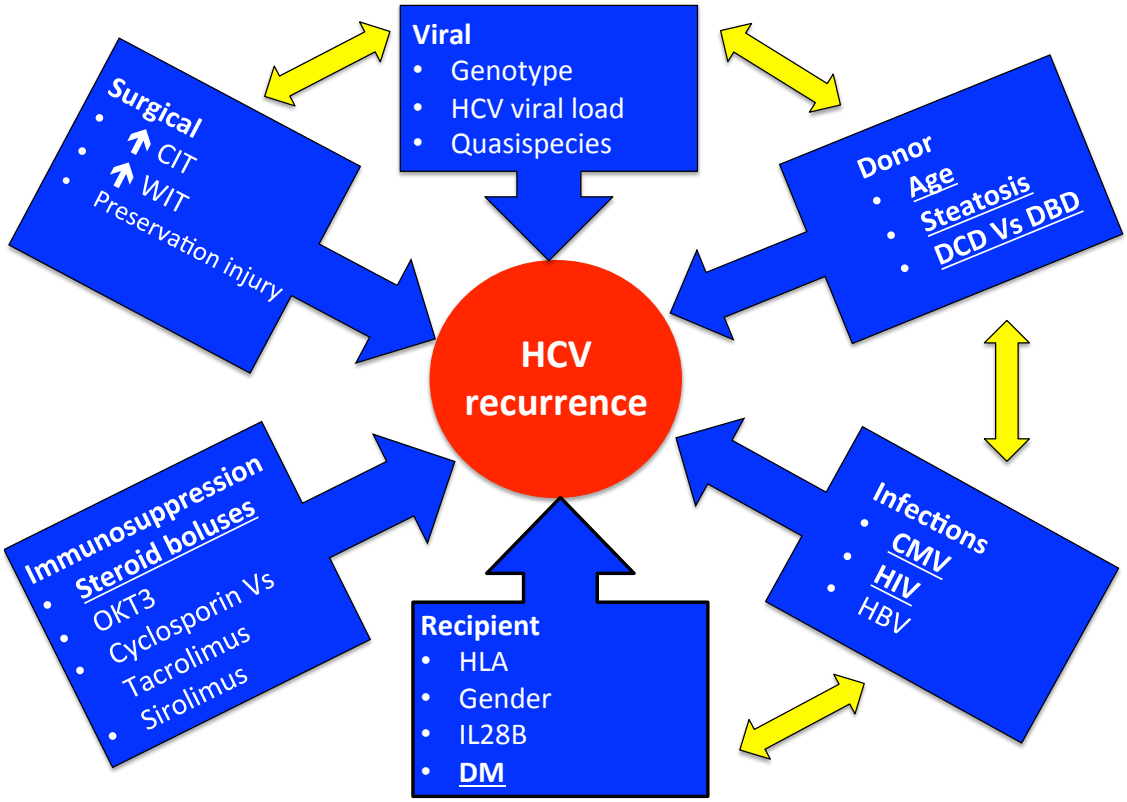


Figure 2.5 Factors influencing HCV recurrence post liver transplantation.

CIT, cold ischaemia time; WIT, warm ischaemia time; HLA, human leukocyte antigen; IL28B, interleukin 28B; DM, diabetes mellitus; CMV, cytomegalovirus; HIV, human immunodeficiency virus; HBV, hepatitis B virus; DCD, donation after cardiac death; DBD, donation after brain death.

5.1 Surgical factors

A prolonged cold-ischaemia time (CIT) and warm-ischaemia (WIT) time have both been associated with severe recurrence of HCV post transplant (Baron et al., 2000, Briceno et al., 2007, Cameron et al., 2006). Experimental data has suggested that sinusoidal endothelial cells are affected more by a prolonged CIT whereas hepatocytes appear to be more susceptible to the effects of a prolonged WIT (Baron et al., 2000, Clavien et al., 1992). Subsequent hepatocyte proliferation would therefore enhance HCV incorporation into new hepatocytes within the new allograft (Baron et al., 2000). One study demonstrated that the risk of severe HCV recurrence within 1-year increased as the WIT increased; 19%, 40% and 65% after 30, 60 and 90 minutes WIT respectively ($p=0.04$) (Baron et al., 2000). A CIT > 12 hours was also associated with reduced graft survival at 12 months (51% versus 75%, $p<0.0001$). In the same study, on multivariate analysis a CIT > 12 hours was associated with an increased risk of graft failure (OR 7.0, $p=0.034$) (Baron et al., 2000).

5.1.1 Preservation injury

Preservation injury is also known as harvesting or reperfusion injury. Abnormal liver enzymes in the immediate post-transplant period is the manifestation of preservation injury but the diagnosis is usually made histologically (Figure 1.5). Features include neutrophil infiltration, microvesicular steatosis, hepatatocytes cytoplasmic aggregation which progresses to centrilobular necrosis, hepatocyte swelling and cholestasis. Preservation injury is associated with hepatocyte loss followed by proliferation which is thought to mediate incorporation of HCV (Baltz and Trotter, 2003). A retrospective

study studied the impact of preservation injury in both HCV and non-HCV infected patients (Watt et al., 2006). Preservation injury has been identified as a risk factor for the development of a stage F3/4 and was associated with inferior survival rates at 1 and 3 years post-LT (Watt et al., 2006).

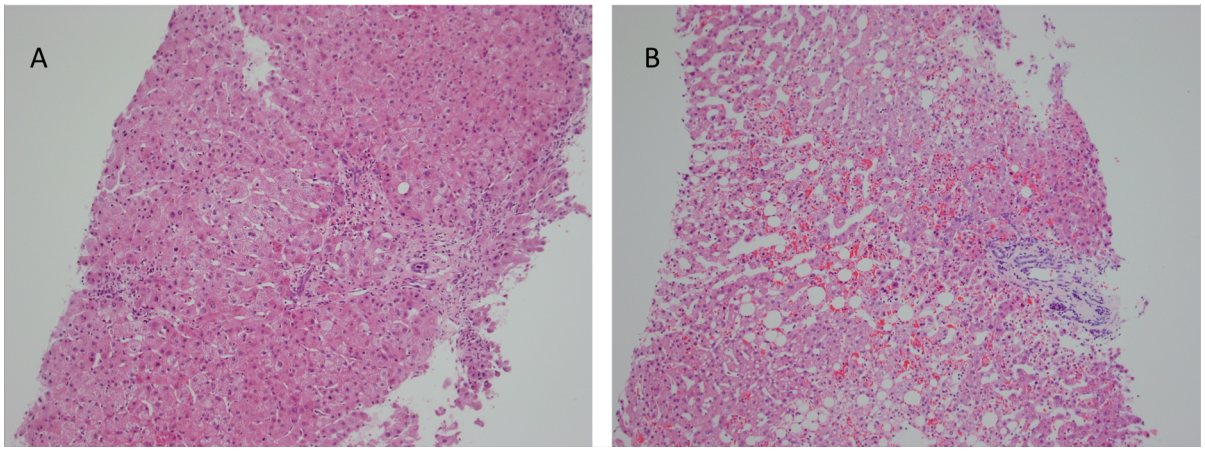


Figure 2.6 Preservation injury (A) mild (no steatosis, no necrosis, no neutrophilic infiltration) and (B) severe (moderate steatosis, hepatocyte loss). H&E stain. Provided by Dr Alberto Quaglia.

5.2 Donor factors

Donor factors are a potential area where modifications are possible. However, given the limited donor pool and availability of suitable grafts, the use of marginal grafts is increasing. The umbrella term of 'marginal donor grafts' or extended criteria donor grafts include donors after cardiac death (DCD), donors infected hepatatotropic viral infections (e.g. HBV or HCV), steatotic grafts, split-livers and organs from elderly donors.

5.2.1 Donor age

One of the most significant risk factors associated with a more rapid and severe histological progression of HCV is increasing donor age (Berenguer et al., 2002, Khapra et al., 2006, Lake et al., 2005, Machicao et al., 2004, Neumann et al., 2004b, Rayhill et al., 2007). The underlying mechanism is due to a combination of a greater susceptibility to cold ischaemia and preservation injury, in addition to the increased likelihood of pre-existing steatosis. A donor age of greater than 50 years compared to a donor age of less than 31 years was associated with an increased likelihood (OR 7.8, p=0.001) of developing severe HCV recurrence within the first 2 years after transplantation(Berenguer et al., 2003). A large US retrospective study demonstrated a linear increase in the risk of graft loss with increasing donor age: 41-50 years, HR 1.67; 51-60 years, HR 1.86; > 60 years, HR 2.21 p<0.001)(Lake et al., 2005).

5.2.2 Steatosis

Steatosis accumulates within the liver as age increases and is therefore one mechanism by which older donor grafts are associated with a worse outcome post transplantation. An increased likelihood of initial poor graft function in donor allografts with severe donor macro-vesicular steatosis was demonstrated compared to donor allografts with moderate steatosis (35% vs 17%, p<0.03)(Verran et al., 2003). A study of 51 patients however failed to identify any accelerated fibrosis rates in HCV patients that received a steatotic graft (Burra et al., 2009).

5.2.3 Donor risk index (DRI)

The donor-risk index (DRI) comprises of eight donor and graft characteristics that are significantly and independently associated with increased failure of deceased donor liver transplants (Feng et al., 2006). The DRI allows for an objective and quantitative assessment of relative risk of graft failure relative to a 'standard' donor. A DRI value of greater than or equal to 1.7 was defined as an extended criteria donor graft (Maluf et al., 2009). The DRI has been shown to be a sensitive predictor of graft and patient survival in HCV patients (Maluf et al., 2009).

5.2.4 Donation after cardiac death (DCD) vs. donation after brain death (DBD)

In donation after cardiac death (DCD) livers, death is declared on the basis of cardio-vascular criteria as opposed to brain function. Given the increasing experience in the use of DCD livers, attention has focused on the use in HCV patients but data appears to be conflicting. Initial single centre studies reported poorer outcomes using DCD livers compared to the use of DBD livers (Nguyen et al., 2009, Yagci et al., 2008). Recent studies however have been more encouraging with no long-term differences in patient or graft survival or time to the development of fibrosis stage ≥ 2 (Taner et al., 2011, Tao et al., 2010). Although the largest study, using the UNOS database demonstrated inferior graft survival amongst DCD HCV patients in comparison to DBD HCV patients, graft survival between DCD HCV positive and DCD HCV negative patients were comparable (Uemura et al., 2012).

5.3 Viral factors

5.3.1 HCV genotype

It remains unclear as to whether HCV genotype affects the fibrosis recurrence post-LT. Available data suggests an aggressive pattern of disease recurrence amongst patients with genotype 1 disease, in particular 1b (Di Martino et al., 1997, Feray et al., 1995, Feray et al., 1999, Ghobrial et al., 1999, Prieto et al., 1999). Mechanisms suggested include increased DNA fragmentation, Fas-mediated liver cell apoptosis and increased antigen expression (Di Martino et al., 1997). Data however from the US does not support the notion of a more aggressive recurrence in genotype 1b patients but maybe due the decreased prevalence of genotype 1b disease compared to European populations (Gayowski et al., 1997, Silini et al., 1996). A more rapid fibrosis progression rate amongst patients with genotype 4 disease (1.51 versus 0.95) with a reduced time to cirrhosis has also been reported (Wali et al., 2003). At present, no clear conclusions of the effect of HCV genotype on HCV recurrence rates post-transplant can be made.

5.3.2 HCV viral load

Individuals entering transplantation with active HCV replication will develop recurrent HCV viraemia post transplantation. During the anhepatic phase, HCV RNA viral titres decrease but re-infection occurs during reperfusion of the liver allograft. Initially, viral titres continue to decline due to uptake of HCV into hepatocytes within the allograft before viral titres return to pre-transplant levels at approximately 72 hours. In the majority of patients viral titres exceed the pre-transplant levels (Fukumoto et al., 1996). Individuals with high HCV viral loads (>800,000 IU/ml) at the time of LT are

more likely to have higher viral loads in the post-transplant period with good correlation between liver tissue and serum (Sreekumar et al., 2000, Terrault et al., 1997). HCV viral loads at 4 months post-transplant was identified as sensitive predictor of histological fibrosis at 3 years post-transplant whilst another demonstrated the higher HCV viral loads at 1 year in patients with more advanced fibrosis (Papatheodoridis et al., 1999, Sreekumar et al., 2000).

5.3.3 Quasispecies

The HCV genome has significant genetic heterogenieity due to the accumulation of mutations during viral replication which arises due to the inability of the RNA polymerase to proof-read. The E2 protein region is particularly susceptible to this. This tendency to mutation has therefore led to a survival advantage in evading the host immune response. The role of viral quasispecies in recurrent HCV post-transplant has also been studied and are thought to occur due to pressure exerted on the host immune system in part by the iatrogenic introduction of immunosuppression (Doughty et al., 2000b, Doughty et al., 2000a). Following transplantation, there is a significant decrease in genetic diversity resulting in a more homogenous viral population up until the fourth week post-transplant (Feliu et al., 2004). These very early changes in quasispecies composition suggest entry of HCV into hepatocytes generate a 'bottleneck' effect by selecting certain variants from the viral population (Feliu et al., 2004). Two further studies demonstrated that immediately post-transplantation, viral quasispecies complexity also decreases and remains low in patients that develop an aggressive fibrosing cholestatic variant (Doughty et al., 1998, Doughty et al., 2000a,

Lyra et al., 2002). In a study of 11 patients, those that developed mild fibrosis had greater heterogeneity compared to patients with severe fibrosis (Lyra et al., 2002).

5.4 Infections

5.4.1 Cytomegalovirus (CMV)

Cytomegalovirus (CMV) has been associated with reduced graft survival and a more aggressive fibrosis progression rate in patients with HCV post LT (Bosch et al., 2012, Neumann et al., 2004b, Razonable et al., 2002, Rosen et al., 1997). Different mechanisms of how CMV propagates HCV disease have been postulated and include increase tumour necrosis factor levels, increased ICAM-1 expression and increased immunological stimulation through MHC Class II molecules (Ballardini et al., 1994, Granot et al., 2001, Smith et al., 1992, Tilg et al., 1991). CMV viraemia may also be a surrogate marker of over-immunosuppression. CMV prophylaxis is recommended in high-risk cases (recipient IgG negative, donor IgG positive).

5.4.2 HIV co-infection

HIV co-infection is a recognised risk-factor for a rapid HCV recurrence and FCH (Safadi et al., 2004, Joshi et al., 2011, Antonini et al., 2011). HCV recurrence in HIV positive patients is more aggressive post transplant translating into inferior outcomes compared to HCV mono-infected patients (Terrault et al., 2012, Miro et al., 2012, Duclos-Vallee et al., 2008) Mechanisms for this accelerated fibrosis rate amongst HIV/HCV co-infected patients remain undelineated but extrapolation from data in HIV/HCV co-infected patients pre-transplant would suggest a prominent role of a

weakened adaptive immune response, reduced ratio of $CD4^+$ to $CD8^+$ cells, reduced IL-10 production, increased microbial translocation, and insulin resistance (Garba et al., 2002, Safadi et al., 2004, Balagopal et al., 2008). Along these lines, recent evidence suggests that Impaired $CD4^+$ T cell stimulation of NK cell anti-fibrotic activity may contribute to accelerated liver fibrosis progression in HIV/HCV patients (Glassner et al., 2013). In addition, HIV gp120 protein has been shown to modulate different aspects of HSC biology, including directional cell movement and expression of pro-inflammatory cytokines (Bruno et al., 2010).

5.4.3 HBV co-infection

3-11% of patients undergoing LT are co-infected with HBV and HCV (Feray et al., 1998, Huang et al., 1996). In post-LT setting, the effect of HBV/HCV co-infection appears to be conflicting. Improved survival rates were associated with viral co-infection but only 9 patients with HBV/HCV were included in the study (Rifai et al., 2007). A larger study of 652 patients however, demonstrated a milder course of HCV recurrence in patients with HBV co-infection (Feray et al., 1999). A recent study using the UNOS database, demonstrated superior graft survival in the HBV/HCV co-infected group compared to the HCV mono-infected group (84% at 1 year and 54% at 10 years vs 83% 1-year survival and 46% 10-year survival (Waki et al., 2011). Improved survival amongst HBV/HCV co-infected patients is likely to be due to a combination of HBV prophylaxis and antiviral therapy, and the observation of lower immediate HCV viral loads (Waki et al., 2011).

Hepatitis B core antibody (HBcAb) positive grafts have been used successfully in HCV positive patients (Jain et al., 2005, Rayhill et al., 2010). Data is conflicting with regards to graft survival outcomes. Recent data has suggested inferior graft survival rates amongst HCV-RNA positive recipients who received HBcAb positive donor grafts (Tandoi et al., 2012). Prophylaxis with anti-HBV therapy is recommended in this group.

5.5 Recipient factors

5.5.1 Human leukocyte antigen (HLA)

Given the integral role of HLA class I and class II antigens in the recognition of HCV peptides, studies have evaluated the role of donor recipient HLA mismatch on recurrence rates of HCV post-transplant. One study failed to identify any relationship between histological outcome and the extent of matching of either HLA-DR or DQ (Gane et al., 1996a). A more recent German study demonstrated a reduced number of cases of rejection associated with a decreasing numbers of HLA mismatches ($p < 0.05$) but no effect of HLA-A, -B or -DR matching on the number of recurrent or steroid resistant rejection episodes (Langrehr et al., 2006). In the same study, HLA-A and –DR matching had no effect on fibrosis progression post transplant but HLA-B matching correlated significantly with an accelerated rate of fibrosis at 1-year (Langrehr et al., 2006). Subsequent studies have however demonstrated that HLA-DRB1 donor/recipient mismatch, in particular fully mismatched donor-recipients, was related to histological recurrence (Belli et al., 2000). HCV fibrosis progression was associated with the frequency HLA-DRB*11 and HLA-B14 alleles (Belli et al., 2000). Patients with advanced fibrosis (F4-6) had an increased frequency of HLA-DRB1*03 and HLA-B14

alleles but a reduced frequency of HLA-B12 and DRB1*11 in comparison to patients with mild fibrosis (F1-3). Full DRB1 mismatch remained a significant predictor of severe recurrence on multivariate analysis (HR 2.9, p=0.002)(Belli et al., 2000).

5.5.2 Genetic polymorphisms

The recent discovery of single nucleotide polymorphisms (rs12979860 and rs8099917) near the IL-28B gene, which encodes IFN-λ3, are associated with the prediction of treatment success with PEG-IFN and ribavirin irrespective of race (Ge et al., 2009). The CC genotype is associated with the greatest chance of SVR, followed by the CT and TT genotypes (Ge et al., 2009). IL-28B polymorphisms are now regarded as the strongest pre-treatment predictor of SVR in genotype 1 infected patients (Ge et al., 2009, Stattermayer et al., 2011).

There are 3 members of the type III IFN- λ family: IL-29, IL-28A and IL-28B. They are functionally IFNs, structurally are very similar to IL-10 and located on chromosome 19 (Gad et al., 2009, Kotenko et al., 2003, Sheppard et al., 2003). The most studied single-nucleotide polymorphisms (SNPs) in response to IFN- therapy, rs12979860 and rs8099917 are upstream of IL28B and IL28A. These IFN-λs are triggered by viral infections and then induce antiviral as well as antitumor activity through the innate and adaptive immune system pathway (Yoshimoto et al., 2011, Morrow et al., 2010). Both IFN-α and IFN-λ induce signalling through the Jak-STAT (Janus kinase signal transducer and activator of transcription) pathway which then activate IFN-stimulated genes (ISGs) (Kotenko et al., 2003, Zhou et al., 2007). IFN-α binds to type I IFN receptors resulting in an early expression of ISG followed by a rapid decline (Marcello

et al., 2006). In contrast, IFN- λ binds to IL-10 and IL28 receptors expressed on hepatocytes, epithelial cells and plasmacytoid dendritic cells (Kotenko et al., 2003, Sommereyns et al., 2008). The result this time is ISG expression albeit a weaker one but one which increases steadily over time (Zhou et al., 2007). The NF-κB pathway appears to be integral in the IFN- λ mediated activation of STAT (Iversen et al., 2010).

Genome wide associated studies (GWAS) permit screening for representative SNPs which may be associated with specific disease phenotypes or response to treatments. However, GWAS require large study sample sizes in order to detect these changes which are often cumulative. The first publication was performed in 1671 patients who were part of the IDEAL study (McHutchison et al., 2009) (Ge et al., 2009). The cohort consisted patients of European ancestry, African-Americans and Hispanics. The authors identified the rs 12979860 SNP which was located in a noncoding region 3kb upstream of IL-18B to be strong predictor of SVR to PEG-IFN –α and ribavirin (Ge et al., 2009). Patients homozygous for the major allele, the favourable genotype rs12979860 CC were more twice as likely to achieve an SVR compared to patients with less favourable genotypes (CT > TT) ($p=1.37 \times 10^{-28}$) (Ge et al., 2009). The positive predictive value of the CC genotype was evident across all patient groups (Europeans, $p=1.06 \times 10^{-25}$; African-Americans $p=2.06 \times 10^{-3}$; Hispanics $p=4.39 \times 10^{-3}$). The response to anti-viral therapy across the different patient cohorts helped explain the inferior SVR rates in African-Americans observed in this study. Subsequent studies, performed in Japan and Australia identified the rs8099917 genotype (TT > GT > GG) (Tanaka et al., 2009, Suppiah et al., 2009). The rs8099917 SNP is located in the downstream flanking region, the third intron and the upstream flanking region of IL28B. Real-time quantitative PCR

in peripheral blood mononuclear cells also demonstrated significantly lower levels of IL28B mRNA expression in patients with the minor alleles (GT and GG) (Tanaka et al., 2009).

As well as being the strongest pretretament predictor of SVR, the IL28B SNP has also been evaluated in patients that are able to resolve acute HCV and thereby clear the infection. Two studies which evaluated the rs8099917 SNP and the rs12978860 SNP respectively and identified the favourable genotypes (rs8099917 TT and rs12978869 CC were strongly associated with spontaneous clearance (Thomas et al., 2009, Rauch et al., 2010). The favourable rs12979860 CC genotype is also associated with spontaneous clearance in children that have been infected via vertical transmission (Ruiz-Extremera et al., 2011).

The SNPs in IL-28B (IFN- λ3) have been studied in the post-LT both in the donor and recipients in order to predict HCV graft recurrence(Lange et al., 2011, Eurich et al., 2011, Charlton et al., 2011, Eurich et al., 2012, Duarte-Rojo et al., 2012, Veldt et al., 2012). IFN-λ plays an integral role in the modulation of the adaptive immune system as well increasing production of IP-10 (interferon-gamma inducible protein – 10). Unsurprisingly patients that required liver-transplantation were more likely to carry the less favourable rs12979860 CT or TT genotype compared to the non-transplanted patients (Lange et al., 2011). There is however conflicting evidence with regards to the predictive value of the IL-28B SNPs on HCV fibrosis rates post- transplant which may be partly due to the heterogeneity of the patient cohorts. One study demonstrated a

delayed time to recurrence of HCV with the rs12979860 CC IL-28B genotype compared to those with CT and TT genotypes at 5 years (78% vs. 87% vs. 100%, p=0.02) and another study demonstrated a delayed time to the development of advanced fibrosis (CC = 64.9 months, CT 45.8 months, TT 30.0 months, p=0.02) (Eurich et al., 2012). Multivariate Cox regression analysis demonstrated that the recipient IL-28B rs12979860 CC allele was an independent predictor of delayed recurrence of HCV (defined as detectable HCV RNA in serum and allograft histology demonstrating a lymphocytic infiltrate in the absence of ACR) at 2 years (HR 0.62 p=0.008) and 5 years (HR 0.63, p=0.003)(Charlton et al., 2011). The relationship between recipient IL-28B genotype and time to recurrence of HCV at 2 and 5 years remained independent of donor IL-28B genotype (p=0.03 and p=0.015 respectively) (Charlton et al., 2011). Another study, however, demonstrated no correlation between the stage of fibrosis and rs8099917 IL-28B genotype although a higher median histological grade of inflammation was demonstrated (3.0 for GG, 2.5 for GT and 2.0 for TT, p<0.001) (Eurich et al., 2011). Donor rs12979860 IL-28B genotype does not appear to be predictive of time to recurrence of HCV and possession of the IL-28B CC genoytype in the liver donor did not protect from severe HCV recurrence (Charlton et al., 2011, Cisneros et al., 2012).

5.5.3 Diabetes mellitus and insulin resistance

Diabetes alone or the combination of diabetes and donor age > 55 years are independent predictors of progression to bridging fibrosis/cirrhosis post-LT (Foxton et al., 2006). There does not however appear to be a difference in the progression to

advanced fibrosis between patients with pre-transplant diabetes and those with new onset diabetes after transplantation (NODAT) (Veldt et al., 2009). HCV patients post-transplant with IR (HOMA-IR>2.5) were found to have a higher fibrosis rates at 1, 3 and 5 years compared to patients with normal insulin sensitivity and IR was identified as an independent risk factor for developing advanced fibrosis (Veldt et al., 2009).

5.6 Immunosuppression

5.6.1 Corticosteroids

OKT3, an anti-CD3 monoclonal antibody, and ATG (rabbit anti-thymocyte globulin) are strong risk factors for HCV recurrence, cirrhosis and graft failure(Neumann et al., 2004b). Use of corticosteroids for acute cellular rejection (ACR) results in increased HCV RNA levels(Gane et al., 1996a, Garcia-Retortillo et al., 2002, Charlton and Seaberg, 1999). Two randomised trials of steroid-free induction using daclizumab (IL-2 blocker) failed to demonstrate any benefit in the reduction of mean fibrosis stage at 1-year post-transplant (Kato et al., 2007, Klintmalm et al., 2007).

The rate of withdrawal of corticosteroids appears to be a contentious issue. Initial reports suggested that early withdrawal was associated with improved outcomes (Everson, 2002). However other earlier studies demonstrated a more rapid recurrence of HCV in patients who had a rapid withdrawal of corticosteroids (Belli LS et al. 2003; Brillanti S et al. 2002). A more recent prospective study, confirmed the beneficial effect of a slow tapering of corticosteroids (> 3 months)(Vivarelli et al., 2007). A balance with regards to immunosuppression needs to be achieved in HCV patients to avoid over-

immunosuppression and episodes of ACR, both of which promote HCV-related disease progression.

5.6.2 Calcineurin inhibitors

Ciclosporin in-vitro has been shown to demonstrate a suppressive effect on HCV replicon RNA levels and HCV protein expression as well as inhibiting the multiplication of the HCV genome in a human hepatocyte HCV infected cell-lines (Watashi et al., 2003). In vivo data has also demonstrated a modest decline in HCV RNA titres in switching patients to ciclopsorin from tacrolimus (Firpi et al., 2010). These data, along with ciclosporin's inhibition of NS5B binding to cyclophilin B, an integral part in HCV viral assembly, might suggest ciclopsorin be the immunosuppressant of choice. (Watashi et al., 2003, Watashi et al., 2005, Hirano et al., 2008, Nakagawa et al., 2005). However this is not confirmed by clinical studies (Berenguer et al., 2007, Levy et al., 2006, Berenguer et al., 2010). A meta-analysis also highlighted the use of tacrolimus to be associated with improved patient survival (Berenguer et al., 2007). At present, available data would not suggest any benefit of the use ciclopsorin-based immunnosuppression over tacrolimus in patients with HCV with regards to fibrosis progression.

5.6.3 Sirolimus

In vitro studies and animal studies have shown that sirolimus inhibits collagen production by HSCs (Akselband et al., 1991, Neef et al., 2006). Although sirolimus has been associated with a lower incidence of advanced fibrosis (F≥2) at 1 year significant

side effects are described leading to early discontinuation (McKenna et al., 2011). No overall patient survival however, was conferred with the use of sirolimus. Another important finding from this study is that nearly half of the patients that received de novo sirolimus had to stop due to impending surgery or side effects (i.e. mouth ulcers, cytopenias). At present, further prospective studies are required to validate the benefits of sirolimus in attenuating fibrosis progression in HCV patients post-transplant.

5.6.4 Other agents

Initial data identified MMF (mycophenalate mofetil) use as a potential risk factor for recurrent HCV disease, but no clear association has been demonstrated in more recent studies (Berenguer et al., 2003, Wiesner et al., 2005). Interleukin-2 receptor blockers do not appear to impact negatively on HCV recurrence (Klintmalm et al., 2007). Recent data suggests that azathioprine in combination with tacrolimus delays fibrosis progression although the mechanism of action appears unclear (Manousou et al., 2014). Mechanisms for the attenuated fibrosis progression in azathioprine group remain unclear.

6. Assessment of liver fibrosis and progression post-transplant

6.1 Liver biopsy

Liver biopsy remains the 'gold standard' to assess fibrosis in patients with recurrent HCV post LT (Bain et al., 2004). The Ishak scoring system is the most widely used but other commonly used criteria include the Metavir and Sheuer (Ishak et al., 1995). A one-year protocol liver biopsy can stratify fibrosis progression. In one study, 13% of patients with moderate fibrosis (F2-3, Ishak) at 12 months progressed to cirrhosis within 3 years compared to no patients with minimal fibrosis ($p<0.0001$) (Firpi et al., 2004). In the same study, 14% of patients with minimal fibrosis on their 12 month biopsy developed advanced fibrosis (F>4, Ishak) compared to 34 % of patients with moderate fibrosis on the 12 month biopsy ($p=0.02$)(Firpi et al., 2004). Other benefits of liver biopsy include the ability to identify and exclude other causes of liver dysfunction in the post-transplant period. Limitations of the use of liver biopsy include sampling variability and both inter- and intra-observer variability (Bedossa et al., 2003, Westin et al., 1999). Measurement of the hepatic venous pressure gradient (HVPG) has also been used in the post-transplant setting (Samonakis et al., 2007). One study deemed it to be more accurate than liver biopsy in identifying patients at risk of clinical decompensation (Blasco et al., 2006). A HVPG > 6 mmHG 1 year post transplant appears to identify patients with HCV recurrence that are likely to decompensate (Samonakis et al., 2007, Blasco et al., 2006).

Histological scores listed above are categorical systems that provide a description of the architectural changes and the sites of fibrosis. The collagen proportionate area

(CPA) is a quantitative assessment of collagen deposition using computer-assisted digital image analysis. It utilizes picro Sirius red stained histological sections to quantify liver collagen and therefore fibrosis (Standish et al., 2006, Bedossa et al., 2003). The quantity of bound stain correlates with the chemically determined collagen content. CPA does not interfere with other evaluations and excludes collagenous structures irrelevant to the disease process (Standish et al., 2006). CPA correlates with Ishak stage, HVPG and the CPA at 12 months post-LT is predictive of clinical outcome (Calvaruso et al., 2009, Manousou et al., 2011). The rate of CPA increase (CPA fibrosis progression rate) also appears to be predictive of clinical outcome post –LT (Manousou et al., 2013).

6.2 Laboratory based tests

6.2.1 Indirect markers

The inherent risks of liver biopsy have led to the development of non-invasive markers of fibrosis. The 'aspartate aminotransferase (AST)-to-platelet' ratio index (APRI) has been studied extensively pre-transplant(Shaheen and Myers, 2007, Lin et al., 2011). In a study of 51 patients, using AUROC (area under receiver operating characteristic) curves, the APRI was able to predict fibrosis (0.8, $p<0.0001$) with an optimal cut-off of >1.4 to predict fibrosis stage > F2 (sensitivity 76%, specificity 77%, positive predictive value 46% and negative predictive value 93%)(Toniutto et al., 2007). Another study identified 4 variables on multivariate analysis (prothrombin time, albumin/total protein ratio, AST and time from liver transplantation that were associated with fibrosis progression (Benlloch et al., 2005). These parameters were then used to

construct a formula (Benlloch score) that was predictive of fibrosis progression in both a training and validation set (AUROC 0.8 and 0.85 respectively) (Benlloch et al., 2005).

6.2.2 Direct markers

One study evaluated the combination pro-peptide of type III pro-collagen (PIIINP), hyaluronic acid (HA) and tissue inhibitors of matrix metalloproteinases (TIMP-1) to develop the 3-M-ALG algorithm {discriminant score = -7.412 + [ln (HA) x 0.681] + [ln (PIIINP) x 0.775] + [in(TIMP-1] x 0.494} (Carrion et al., 2010a). Recordings were taken at 3, 6 and 12 months post-transplant and compared to HVPG and histological scoring on liver biopsy at 12 months. AUROC analysis for identifying F ≥2 for 3-M-ALG at 3, 6 and 12 months were 0.67, 0.77 and 0.78 in comparison to 0.75, 0.87 and 0.90 for HVPG(Carrion et al., 2010a). The 3-M-ALG score demonstrated a greater diagnostic ability than the APRI, AST/ALT ratio and the Benlloch score. Patients with a 3-M-ALG score ≥ 2 also had an increased risk of developing an episode of clinical decompensation (Carrion et al., 2010a). The London Transplant Centre (LTC), derived from the King's Score incorporates time from liver transplantation, AST, INR and platelet count (Cross et al., 2010, Cross et al., 2009). The LTC AUROC (0.8) for advanced fibrosis (Ishak F>4) appeared better than the King's score (0.73) and the APRI (0.73)(Cross et al., 2010). The FibroTransplant score, another derived model for the prediction of advanced fibrosis post-transplant, performed as well as the Benlloch score, APRI, and Forns index (Beckebaum et al., 2010).

6.3 Transient elastography

The most studied non-invasive measurement of fibrosis is transient elastography (TE) and allows a longitudinal assessment. It is important to emphasize that cut-off values are different to non-transplant patients. In a study of 95 patients with recurrent HCV post LT, a LS value of 5.6kPa was representative of F 0-1 (Ishak), 7.6kPa representative of F2-3 and 16.7kPa representative of F4-6(Rigamonti et al., 2008). AUROC analysis demonstrated a cut-off of 7.9kPa to be representative of F≥3 (0.85, 95% CI 0.76-0.92)(Rigamonti et al., 2008). In another study TE was evaluated at 3, 6, 9 and 12 months post-transplant in comparison to a 12month post-transplant liver biopsy (Carrion et al., 2010b). Their results demonstrated statistically different TE readings at 3, 6, 9 and 12 months between patients with slow fibrosis progression (6.9, 6.9, 7.5, 6.6 kPa) compared to patients with rapid fibrosis progression (7.5, 9.9, 9.5, 12.1 kPa, $p < 0.01$). LS measurements of between 8.7-9.9 kPa appear to be representative F > 2 in patients post transplantation with recurrent HCV(Carrion et al., 2010b, Harada et al., 2008). A LS value of ≥ 8.7kPa at 12 months was associated with an increased risk of hepatic decompensation 5 years post LT (47% vs. 8%, $p<0.001$) and an increased risk of graft loss (Crespo et al., 2014). Comparative studies have been performed to assess the utility of TE and laboratory based markers(Harada et al., 2008, Beckebaum et al., 2010). One study of 56 patients found that serum HA (AUROC 0.52), type-4 collagen (AUROC 0.62), ALT (AUROC 0.64) and APRI (AUROC 0.70) were all inferior to TE in predicting F≥2 (AUROC 0.92)(Harada et al., 2008).

6.4 Magnetic resonance elastography (MRE)

MRE utilises acoustic shear waves that pass through the liver and generates propagation patterns which provide quantitative stiffness values of the entire liver(Manduca et al., 2001). MRE has been evaluated in the pre-LT setting demonstrating a good ability to distinguish between normal and fibrotic livers(Rouviere et al., 2006, Talwalkar et al., 2008). MRE appeared to be a sensitive tool to detect fibrosis post-LT in one small study(Lee et al., 2011). In the same study, falsely elevated values were noted in cases with alterations in regional blood flow, oedema, inflammation, portal vein thrombosis and rejection (Lee et al., 2011). At present, no standardized cut-off values for MRE exist for the different stages of fibrosis.

6.5 CXCL10

Given the integral role of the innate and adaptive immune system in the pathogenesis of HCV infection, both chemokines and cytokines have been suggested as possible markers of treatment outcome. Interferon-gamma inducible protein 10kDa (IP-10) or CXCL10 is a potent chemokine which plays an important role in the pathogenesis of HCV infection (Qin et al., 1998, Zeremski et al., 2007). CXC chemokines can be divided into two subgroups according to the presence of the ELR motif or Glu-Leu-Arg: ELR^+ CXC chemokines are pro-angiogenic compared to ELR^- chemokines such as CXCL10 which demonstrates angiostatic properties (Belperio et al., 2000). CXCL10 binds the chemokine (C-X-C motif) receptor 3 (CXCR3) which is a seven trans-membrane receptor coupled to G proteins. CXCL10 is secreted by CD4+, NK, NK-T cells, neutrophils, monocytes, endothelial cells, fibroblasts, thyrocytes, hepatic stellate cells ,

preadipocytes and is dependent on IFN-γ, which in turn is dependent on IL-12 (Engel and Neurath, 2010). CXCL10 levels are a marker of the host immune response in particular the T helper (Th) 1 response. The activated and recruited Th1 lymphocytes are responsible for the increased IFN-γ and TNF-α production which results in increased CXCL10 secretion through an amplification feedback loop (Antonelli et al., 2008).

CXCL10 is induced in hepatocytes in chronic infection through the recognition of conserved PAMPS (pathogen associated molecular patterns) by innate pattern recognition receptors including TLR3 and retinoic acid inducible gene 1 (RIG-1) (Beinhardt et al., 2012, Sumpter et al., 2005). TLR3 and RIG1 both sense HCV infection, TLR3 in particular recognises double stranded RNAs generated during viral replication (Wang et al., 2009, Li et al., 2012, Saito et al., 2008). The resultant activated TLR3 binds TRIF (TIR-domain containing adapter inducing IFN-β which activates a variety of transcriptional factors (IRF3, IRF7, NF-κB) which induce pro-inflammatory cytokines/chemokines and type I and type III IFNs(Takeuchi and Akira, 2010, Osterlund et al., 2007). Type I IFNs bind to IFNAR1/IFNAR2 receptors which activates Janus kinases and STAT proteins which in turn induce ISGs (Ank et al., 2006, Aaronson and Horvath, 2002)

Concentrations of CXCL10 are elevated in HCV infection compared to healthy controls and it is an important chemo-attractant for $CD4^+$ lymphocytes, monocytes and natural killer (NK) cells but not for $CD8^+$ lymphocytes or neutrophils (Taub et al., 1993, Taub et al., 1995). Serum levels of CXCL10 are reflective of hepatic IP-10 mRNA and predict HCV RNA first phase decline during anti-viral treatment (Askarieh et al., 2010). Decreased pre-treatment levels of CXCL10 have been previously associated with SVR in HCV mono-infected, 'difficult to treat' HCV mono-infected and HIV/HCV co-infected cohorts (Lagging et al., 2006, Reiberger et al., 2008, Romero et al., 2006). High CXCL10 levels are associated with failure of spontaneous HCV clearance in acute infection (Grebely et al., 2013). Data in patients with acute and early chronic HCV infection suggests that CXCL10 levels were predictive of early viral kinetics but not of the ultimate treatment outcome (Feld et al., 2013).

The role of hepatic gene expression, in particular interferon-stimulated genes (ISG) has been studied pre- and post- anti-viral therapy in order to establish host factors which may be associated with treatment response (Feld et al., 2007, Sarasin-Filipowicz et al., 2008). Animal models have also demonstrated that interferon non-response is associated with pre-activated, either maximally or inappropriately stimulated intrahepatic ISG expression (Asselah et al., 2008, Chen et al., 2005). Induction, of already high baseline ISGs, by exogenous administration of interferon is therefore associated with a poor therapeutic response.

CXCL10 is a valid marker of ISG activation, lower levels associated with a lower level of activation of the innate, interferon associated immune response (Chen et al., 2005, Sarasin-Filipowicz et al., 2008). CXCL10 is also produced by liver sinusoidal endothelial cells and hepatic stellate cells (Harvey et al., 2003, Holt et al., 2009, Shields et al., 1999). CXCR3 is highly expressed in IL-2 activated T lymphocytes but is undetectable in resting T lymphocytes, B lymphocytes, monocytes or granulocytes (Jinquan et al., 2000). CXCR3 is highly expressed on activated effector cells and is closely linked to the Th1-type immune response indicating its integral role in the recruitment of activated lymphocytes to the inflamed liver (Qin et al., 1998). Given that CXCL10 is produced by endothelial cells, monocytes and by HSCs attention has therefore turned to assessing the role of CXCL10 in predicting fibrosis progression. It remains unclear as to why lower CXCL10 levels are associated with a successful treatment response. CXCL10 gene expression is increased after exposure to interferon, and therefore one mechanism of action postulated is that CXCL10 and hence ISGs are up-regulated and demonstrate a more robust response to the administration of exogenous interferon which results in a higher SVR rate. Another suggested hypothesis is that the elevated CXCL10 levels are in an antagonist or truncated form in non-responders (Casrouge et al., 2011). The antagonist form of CXCL10 is generated due to in situ amino-terminal truncation of the protein and via dipeptidyl peptidase IV (DDP4 or CD26) possibly in combination with other proteases (Casrouge et al., 2011). DPP4 is itself expressed and regulated by T lymphocyte function with high serum DPP4 levels associated with poor treatment outcomes in patients with genotype 1 chronic HCV (Firneisz et al., 2001)

The truncated form of CXCL10 retains CXCR3 binding but does not induce signalling (Casrouge et al., 2011, Proost et al., 2001). Studies have also shown that differences in the expression of hepatic ISGs before treatment are associated with the IL-28B polymorphism (Honda et al., 2010, Urban et al., 2010). The non CC IL-28B genotype exhibited higher ISG levels compared to the CC IL-28B genotype.

6.6 MicroRNAs

MicroRNAs (miRNA) were discovered in 1993 and are small (approximately 22 nucleotides) non-coding RNAs that regulate gene expression at the post-transcriptional level by specifically binding to target messenger RNA (mRNA), leading to their degradation or translational repression (van Rooij and Olson, 2007). miRNAs have been shown to regulate more than one-third of all human genes (Krek et al., 2005). Genes that encode miRNA are transcribed from miRNA genes via RNA polymerase II or III (primary transcript (pri-miRNA)), and then cleaved by DROSHA-DGCR8 complex to make a short precursor (pre-miRNA) (Bartel, 2009). This occurs in the nucleus. The pre-miRNA is then exported into the cytoplasm through the exportin-5 complex. Next, the pre-miRNA is cleaved by DICER and TRBP to create a miRNA duplex (mature miRNA). The most stable aspect of the strand is then selected and loaded with Ago2 and GW182 into the RNA-induced silencing complex. Mature miRNA complentary to the 3' UTR of the target miRNA gene either undergo cleavage, translational repression or activation (Bartel, 2009).

miRNAs repress their target through the interaction with the 3' untranslated region (UTR) and this change is detectable at the RNA level (Ambros, 2004, Bartel, 2009). Conversely, miRNAs that engage their targets in a non-3 UTR dependent fashion cause upregulation of their targets. Multiple miRNAs are able to regulate messenger RNA (mRNA), therefore creating a complex situation with regards to their capacity to modulate various biological processes (Szabo and Bala, 2013). By simultaneously regulating the expression of large numbers of genes, miRNAs have been implicated in controlling diverse biological processes, including apoptosis, proliferation, inflammation and differentiation. They have highly conserved cross species sequence homology (Cummins and Velculescu, 2006).

6.6.1 miRNAs and liver disease

miRNAs have been implicated in a variety of mechanisms which are relevant to progression of liver disease. Liver inflammation is an important mechanism a key mechanism and miRNA-155 induction has been highlighted in mouse models of NASH and alcohol related steato-hepatitis (Cheung et al., 2008, Bala et al., 2011). Increased miR-155 can lead to increased TNF expression which promotes liver inflammation (Bala et al., 2011). Conversely, miRNA-146a acts as a molecular break for inflammation and therefore mice deficient in miRNA-146a develop hyper-inflammation through NF-κB (Zhao et al., 2011).

Other relevant mechanisms include apoptosis, necrosis and cell cycle proliferation. Inhibition of miRNA – 15b and miRNA-16 in a mouse model resulted in a reduction of hepatic apopotosis and TNF production (An et al., 2012). Induction of miRNA-21 has

been shown to inhibit hepatocyte DNA synthesis (Song et al., 2010). Other miRNA implicated in cell profileration include miRNA-26a (down regulated after hepatectomy), miRNA-217 (down regulated during liver regeneration) and miRNA-221 (Pan et al., 2012, Yuan et al., 2013).

miRNA-122 is specifically and abundantly expressed in hepatocytes and accounts for approximately 70% of the total miRNA within the liver (Jopling et al., 2005). miRNA-122 is involved in key hepatic homeostatic mechanisms affecting genes involved with cholesterol and lipid metabolism. Mice deficient in miRNA-122 had increased intrahepatic recruitment of inflammatory cells and pro-inflammatory cytokines, thereby also implicating an integral role in hepatic inflammation (Tsai et al., 2012, Hsu et al., 2012). Mice that are deficient in miRNA-122 develop steatohepatitis and fibrosis, with the miRNA-122a-KLF6 axis playing an integral role (Tsai et al., 2012)(13). Further data from a CCl_4^- induced liver fibrosis mouse model demonstrated a reduction in miRNA-122 in activated HSCs (Li et al., 2013). miRNA-21 has been shown to correlate positively with HCV mediated fibrosis by targeting SMAD7 which enhances TGF-β mediated fibrosis (Marquez et al., 2010). A study performed in HCV infected patients with fibrosis and healthy volunteers demonstrated upregulation of miRNA-20a and miRNA-92a in patients HCV related fibrosis (Shrivastava et al., 2013).

miRNA-122 has been shown to facilitate the replication of HCV RNA by interacting with the 5’ non-coding region of the viral genome. Serum levels have been shown to correlate with serum alanine transaminase (ALT) activity and necro-inflammatory

activity in patients with HCV (Jopling et al., 2005, Bihrer et al., 2011). The role of miRNAs in hepatic fibrosis has been evaluated previously in a mouse model and in human liver biopsies from patients with HCV pre liver transplantation (Murakami et al., 2011). miRNAs have been shown to play an essential role in HSC function, activation and therefore hepatic fibrosis (Moreira, 2007, Atzori et al., 2009). A number of miRNAs have been identified which play an important role in the regulation of hepatic fibrosis. Both profibrotic (miRNA-119a/b, miRNA-34) and antifibrotic (miRNA-29, miRNA-150) miRNAs have been identified (Roderburg et al., 2011, Kwiecinski et al., 2012, Mannaerts et al., 2013, Venugopal et al., 2010).

6.6.2 miRNAs as biomarkers

miRNAs are viewed as attractive, potential biomarkers because of their stable, cell-free form in blood. miRNAs have been demonstrated in different body fluids and can be released in microvesicles, exosomes apopotic bodies or bound to HDL (Weber et al., 2010, Mitchell et al., 2008, Vickers et al., 2011). These circulating miRNAs are resistant to endogenoud RNases in the blood. Critically, what remains unclear is how miRNAs make their way into the blood-stream or into other bodily fluids; whether it is the result of cell death or secondary to active secretion from tissue cells. There is increasing evidence that serum/plasma concentrations of miRNAs are altered in multiple disease pathologies, including HCC (Fan et al., 2008, Kosaka et al., 2010a, Xu et al., 2011). Studies specific to HCV demonstrate that miRNA-122 expression correlates with serum transaminases and necro-inflammatory activity on liver biopsy, but not with fibrosis stage or parameters of liver function in patients with chronic HCV (Bihrer et al., 2011). miRNA-122 levels have also been shown to be decreased in

patients who do not respond to anti-viral therapy compared to responders in the non-transplant setting (Sarasin-Filipowicz et al., 2008). Elevated circulating levels of miRNA-122 have been demonstrated in variety of aetiologies including HCV, HBV, ALD, HCC and NAFLD (Bala et al., 2012, Bihrer et al., 2011, Roderburg et al., 2012, Ji et al., 2011, Tryndyak et al., 2012). It however, remains unclear as to whether the raised levels of miRNA-122 is due to hepatocyte injury alone.

As well as providing diagnostic information, miRNAs provide the possibility of therapeutic intervention. Novel targeted anti-miRNA therapies, so-called antagamirs, have been postulated as future therapeutic targets. In two separate murine models, the introduction of an anti-sense oligonucleotide/antagamir resulted in a reduction of hepatic steatosis and tumorgenesis repression, respectively (Kota et al., 2009). Although the use of antagamirs remains in its infancy, recently published data using a potent inhibitor of miRNA-122 function, has demonstrated a dose-dependent reduction in HCV RNA titres (Janssen et al., 2013). A phase 2a study evaluated miravirsen, a locked nucleic acid modified DNA phosphorothioate antitense oligonucleotide that sequesters mature miRNA-122 in a highly stable hetero-duplex and therefore inhibits its function. In total, 36 patients with genotype 1 HCV were enrolled in a randomised, double-blind, placebo-controlled sequential series, ascending multiple dose-ranging study. Patients received 3mg, 5mg, or 7mg of miravirsen or placebo. The results demonstrated that there was a reduction in HCV RNA in a dose-dependent manner and was sustained beyond the administrative period for miravirsen (Janssen et al., 2013). Some patients were able to achieve undetectable HCV RNA levels and there were no cases of viral resistance. A reduction in serum

transaminases were also observed in addition to a reduction in serum cholesterol levels. Side-effects were minimal. These data are an example of the therapeutic options offered by miRNAs.

7. Treatment of recurrent HCV post-transplantation

At present, AVT remains the only viable therapeutic strategy which can alter fibrosis progression (Bahra et al., 2007, Berenguer, 2002). The benefits of achieving a SVR are clear; improvement in liver fibrosis, lower probability of decompensation and a lower cumulative mortality post-transplantation (Berenguer et al., 2008, Picciotto et al., 2007). It should be emphasized that the role of AVT begins in the pre-transplant period with potential transplant candidates being treated on the waiting list.

7.1 Pre-transplant

AVT should be considered in all patients with Child Pugh (CP) - A (compensated) disease and on an individual case basis in patients with CP-B. Overall, AVT is poorly tolerated by cirrhotic patients, although achieving HCV RNA negativity at transplant decreases re-infection and thus recurrence and so is still utilized by some groups. Given the risk of decompensation and side effects (particularly sepsis), the concept of a low accelerating dosing regimen (LADR) was introduced(Everson et al., 2005). A LADR involves starting patients on reduced doses of PEG/RBV and then increasing the doses every 2 weeks to reach standard or maximally tolerated doses. Data from a multi-

centre US study using PEG/RBV only has suggested that patients need to be treated for greater than 16 weeks pre-LT in order to remain HCV RNA negative post-LT (Everson et al., 2013).

Boceprevir and telaprevir (NS3 protease inhibitors) in combination with PEG/RBV were licensed in 2011 for patients with genotype 1 disease. Registration trial data demonstrated improved SVR rates in treatment naïve (68-75%) and treatment-experienced groups (59-88%) although this was predominately in patients with mild fibrosis (Poordad et al., 2011, Jacobson et al., 2011, Bacon et al., 2011, Zeuzem et al., 2011). Interim, week 16 data from the CUPIC (Compassionate Use of Protease Inhibitors in viral C Cirrhosis) study suggests triple therapy (boceprevir or telaprevir plus PEG/RBV) in treatment-experienced cirrhotic patients, some of whom would be potential transplant candidates, is associated with a high incidence of serious adverse events (40%)(Hezode et al., 2013). This 'real-life' experience in predominately Child-Pugh-A cirrhotic patients showed that triple therapy was poorly tolerated; 58 patients (12%) required early treatment discontinuation, 12 patients (2.4%) developed hepatic decompensation and 6 patients died. Risk factors for severe complications or death were delineated by serum albumin < 35g/L and a platelet count ≤ 100,000/mm^3 (Hezode et al., 2013).

New data on the use of the DAAs in cirrhotic patients is emerging. The NEUTRINO study was a single-group open label study of sofosbuvir (SOF) plus PEG/RBV for 12

weeks in 327 patients (17% cirrhotic) with predominantly genotype 1 disease (Lawitz et al., 2013). SOF, a NS5B polymerase inhibitor, has demonstrated a high barrier to resistance, a favourable safety profile and pan-genotypic action. Reported SVR 12 rates were 80% amongst cirrhotic patients. The FISSION study was a randomized, open label, active control study of SOF and RBV in genotype 2 or 3 patients which reported SVR12 rates of 47% in cirrhotic patients compared to 72% in non-cirrhotic patients (Lawitz et al., 2013).

7.2 Post transplant

Two principal strategies have been adopted; pre-emptive treatment and treatment following evidence of histological recurrence. The current 'standard of care' in the post-transplant period is PEG/RBV for 48 weeks irrespective of viral genotype.

7.2.1 Pre-emptive treatment

Pre-emptive treatment of HCV recurrence post-LT is commenced immediately post-transplantation and is based on the hypothesis that virologic recurrence is universal in all patients. The obvious advantages with this strategy are that HCV RNA levels will be at their lowest and liver fibrosis will be minimal.

Studies to date that have adopted this treatment strategy are both limited and heterogeneous (genotype 1 and non-genotype 1 patients), some using interferon-alpha mono-therapy whilst others have used PEG-alpha in combination with ribavirin

(Mazzaferro et al., 2001, Sheiner et al., 1998, Shergill et al., 2005, Singh et al., 1998, Sugawara et al., 2004). Two randomised, prospective studies that used interferon-alpha mono-therapy, beginning 2 weeks after transplantation, clearly demonstrated that those treated were less likely to develop a recurrent hepatitis than patients who were not treated (Sheiner et al., 1998, Singh et al., 1998). Neither study however, was able to demonstrate any survival benefit in patients that received interferon treatment. In a study of 36 patients, using both interferon-alpha and oral ribavirin for 12 months, the authors were able to demonstrate HCV-RNA clearance in 12 patients (33%) (Mazzaferro et al., 2001). An important observation made by this study was that HCV-RNA clearance was more likely to be achieved by those with lower baseline HCV RNA levels.

More recent studies have used PR and demonstrated SVRs between 8-18% (Chalasani et al., 2005, Shergill et al., 2005). Although the SVR rates were considerably lower than those previously reported, the key message from one of these studies was that pre-emptive AVT was only applicable in 51% of patients and the desirable > 80% treatment dose and > 80% treatment duration was only achieved by a small number of patients (14%) (Shergill et al., 2005). Cytopenias, especially anaemia, post-operative complications and severe debilitation relating to the severity of illness pre-transplantation all limit the applicability of AVT.

7.2.2 Treatment of established HCV recurrence

Studies assessing the efficacy of AVT once histological evidence of hepatitis C recurrence has been established are more numerous and reflect the preferred setting to commence anti-HCV therapy. The reasons for this seem to be multifactorial: a recuperated patient with less co-morbid or post-surgical concerns, reduced risk of acute cellular rejection, better graft function and lower doses of immunosuppression. Earlier studies that evaluated this strategy reported SVR rates of between 12-24% with PEG mono-therapy and PEG/RBV (Angelico et al., 2007, Chalasani et al., 2005). In both studies, however, there was a wide variation in median time from transplantation to treatment (6-96 months) and grade of baseline fibrosis demonstrating the heterogeneity of the patients groups.

Most centres institute AVT once histological evidence of HCV is established, usually more than 12 months post transplantation. In order to establish the optimal timing of when to begin treatment, one study attempted to extrapolate the experience and success of treating acute HCV in pre-transplant cohorts and treat patients in the post-transplant period as soon as acute HCV was detected (Castells et al., 2005, Jaeckel et al., 2001). Inclusion criteria included persistent ALT elevation, HCV-RNA positivity, histological evidence of lobular hepatitis consistent with recurrent HCV and no evidence of acute or chronic rejection, biliary obstruction or ischaemic changes. 24 patients eventually underwent treatment with the interval between LT and histological evidence of HCV recurrence of approximately 4 months. 14 patients (58%) had an

EOTR and 8 (35%) had a SVR. Although side effects were common, asthenia and muscle pain the most frequent, no patient discontinued their interferon treatment.

More recent studies have used PEG-IFN alpha-2b (1.5mcg/kg) or alpha-2a (180ug) and RBV 800-1200mg (Angelico et al., 2007, Castells et al., 2005, Carrion et al., 2007, Dumortier et al., 2004, Oton et al., 2006, Picciotto et al., 2007) (Table 2.3). Reported SVR rates for all genotypes range between 8-45% (Berenguer, 2008, Wang et al., 2006, Xirouchakis et al., 2008). SVR rates for genotype 1 patients however, are considerably lower ranging between 13-33% only (Berenguer, 2008). Once again the study cohorts were heterogeneous with wide variation in the time from LT to the start of treatment and the percentage of patients with advanced fibrosis (Angelico et al., 2007, Carrion et al., 2007, Dumortier et al., 2004, Oton et al., 2006, Picciotto et al., 2007, Hanouneh et al., 2008). Treatment appears to be more efficacious when the histological recurrence of HCV is mild; 48% SVR in patients with mild HCV recurrence versus 19% SVR in patients with severe HCV recurrence (Carrion et al., 2007). One consistent finding however, amongst these studies were the numbers of patients that discontinued treatment early and the poor tolerability. Two studies addressed the potential benefit of long-term maintenance antiviral therapy but failed to demonstrate any clear benefit (Kornberg et al., 2007, Walter et al., 2009).

	Total transplanted (n)*	Treated (n)	N(%) Genotype 1	SVR	Duration of Rx (weeks)	Time to treatment post LT (months)	N(%) with advanced fibrosis	N(%) discontinued secondary to side-effects
(Dumortier et al., 2004)	97	20	16(80%)	45%(9/20)	48	28 (median)	-	14 (20%)
(Castells et al., 2005)	83	24	24 (100%)	-	24	3.8 +/-2.2	-	-
(Oton et al., 2006)	171	55	50 (91%)	G1 – 40% Overall – 44%	48 weeks – G1 24 weeks – G2/3	63 (mean)	18 (33%)	13 (24%)
(Angelico et al., 2007)	-	42**	35(83%)	36%	48	44 (median) range 12-96	-	12 (29%)
(Carrion et al., 2007)	140	81	73(90%)	33% (18/54)	24	15	27(33%)	21 /54 (40%)
(Picciotto et al., 2007)	123	61	53(87%)	28% (17/61)	48 weeks – G1 24 weeks – G2	25 (median) Range 3-131	28 (46%)	8 (15%)
(Hanouneh et al., 2008)	-	53	42 (79%)	35% (19/53)	48	15 (median)	10 (19%)	14 (26%

Table 2.3. Studies evaluating treatment outcomes with pegylated interferon and ribavirin for established recurrent HCV post liver transplantation.

*** Total number of patients transplanted during study period**
**** Compared pegylated interferon and ribavirin versus pegylated interferon role**
SVR, sustained virologic response; Rx, treatment; LT, liver transplant; G1, genotype 1

There is only one prospective, multi-center randomised study published that has attempted to determine which treatment strategy is better and safest (Bzowej et al., 2011). The PHOENIX (Pegasys and Copegus Administered After Liver Transplantation for Hepatitis C) study conducted in North America reported SVR rates of 22% in the pre-emptive group but with discontinuation rates of greater than 40% predominately due to haematological side effects (Bzowej et al., 2011). In the same study, those treated for established recurrence had SVR rates of 21%. This study was unable to delineate which strategy was optimal for the treatment of recurrent HCV infection post transplantation, primarily due to small study numbers. However, a repeat of this

study with the addition of the DAAs may be able to establish the optimal treatment strategy but may also reveal significant tolerability issues.

The overall poorer SVR rates reported in patients post-transplant for HCV are likely due to a combination of factors; 1) the high percentage of patients with genotype 1 disease, (2) high percentage of patients with a previous poor response to AVT, (3) higher baseline HCV viral loads, (4) increased incidence of side effects leading to significant dose reductions or discontinuation, (5) the interaction with immunosuppression and (6) the lack of use of growth factors to support bone marrow function.

7.2.3 Predictors of sustained virologic response with pegylated interferon and ribavirin

Predictors of SVR can be divided into pre-treatment variables and on-treatment variables. Pre-treatment factors associated with a SVR include a low baseline HCV viral-load, HCV RNA < 800,000 IU/ml, younger recipient age, non-genotype 1 disease, shorter length between LT and commencing treatment, donor age < 50 years, low baseline bilirubin levels and mild fibrosis(Berenguer, 2008, Wang et al., 2006, Xirouchakis et al., 2008, Hanouneh et al., 2008, Oton et al., 2006, Berenguer et al., 2009) (Table 2.4). On-treatment predictors include an EVR and a RVR.

Pre-treatment
Low baseline HCV viral load (< 800,000 IU/ml)
Younger recipient age
Younger donor age
Non genotype-1 disease
Shorter duration between liver transplantation and anti-viral treatment
Mild fibrosis (F<2)
rs12979860 IL28B CC genotype
On-treatment
Rapid virologic response (RVR)
Early virologic response (EVR)

Table 2.4. Pre-treatment and on-treatment predictors of sustained virologic response with pegylated interferon and ribavirin post-liver.

The IL-28B genotype has also been evaluated in the post-transplant setting (Charlton et al., 2011, Eurich et al., 2011, Eurich et al., 2012, Lange et al., 2011). The favourable CC (rs12979860) and TT (rs8099917) genotypes are both associated with SVR ($p<0.005$) (Lange et al., 2011, Eurich et al., 2011, Charlton et al., 2011). Both recipient and donor IL-28B genotype have been evaluated with one study suggesting a more dominant role for the donor IL-28B genetic polymorphisms on treatment outcome (Charlton et al., 2011).

Available data suggests that the use of ciclosporin over tacrolimus is associated with an increased chance of SVR (RR 2.0, CI 1.2-3.5, p= 0.02) and a reduced risk of relapse (Relative risk 0.4, CI 0.2-0.9, p=0.02) (Firpi et al., 2006, Firpi et al., 2010, Campos-Varela et al., 2012). Suggested mechanisms include the direct antiviral effect of ciclosporin demonstrated in-vitro and the inhibition of NS5B binding to cyclophilin B (Hirano et al., 2008, Watashi, 2010). Results from ReViS-TC study demonstrated that the use of ciclosporin (Relative risk 1.972, p=0.02) and a longer treatment duration of AVT (Relative risk 1.2, p<0.001) were predictive of a SVR (Campos-Varela et al., 2012).

7.2.4 Side-effects and tolerance

Reduced tolerability due to fatigue, asthenia, pyrexia and the development of cytopenias, in particular anaemia is well described. Side effects appear to be more prominent in patients with a severe hepatitis (Carrion et al., 2007). On multivariate analysis, factors associated with the development of significant anaemia (> 5g/dL) with the use of PEG/RBV only included estimated creatinine clearance (Relative risk=0.951, 0.925-0.978, p<0.001), the use of mycophenolate mofetil (Relative risk = 5.3, 1.4-20.0, p=0.01), ciclosporin (Relative risk = 3.5, 1.4-8.7, p=0.008), baseline HCV viral load > 600,000 IU/ml (Relative risk = 4.8, 1.7-13.5, p=0.003) and baseline haemoglobin values (Relative risk=3.0, 1.9-4.7, p=0.001)(Giusto et al., 2011).

The use of interferon can be associated with the development of immune mediated graft dysfunction (IGD) – an umbrella term for acute rejection (AR), chronic rejection (CR) and plasma cell hepatitis (PCH) (Selzner et al., 2011, Stanca et al., 2007, Wang et al., 2006, Ward et al., 2009). Certainly, in the pre-emptive treatment approach, concerns following the introduction of interferon so soon after transplantation are due to the risk of precipitating ACR or an episode of sepsis. The development of a PCH is associated with poor outcomes (Fiel et al., 2008, Ward et al., 2009). In a recent study, evidence of a PCH on a pre-treatment biopsy was the commonest finding in patients that developed IGD and appears to be an important risk factor for the development of IGD (Levitsky et al., 2012) (Levitsky J et al. 2012). Other risk factors for IGD included previously treatment naïve to IFN based therapy, use of PEG-IFN alpha - 2a, a high pre-treatment alkaline phosphatase and a reduction in immunosuppression prior to commencing AVT (Levitsky et al., 2012). The risk of ACR precipitated by treatment overall, appears to be low (0-5%)(Castells et al., 2005, Oton et al., 2006).

7.2.5 New DAAs post liver-transplantation

Due to the recurrence of HCV and difficulty in offering re-transplantation in the context of finite donor resource and increasing demand for transplantation, several teams have utilized PI based triple therapy in the post transplant population. This has been primarily driven by clinical need with several authors reporting results. However, given the first DAAs significant drug-drug interactions (DDI) it *further* increases the complexity of triple based therapy. Both boceprevir and telaprevir inhibit the P450 3A cytochrome and therefore require a reduction in ciclosporin and tacrolimus dosing

(Garg et al., 2011, Coilly et al., 2012). Initial opinion was cautious given a 76 fold increase in tacrolimus exposure with concomitant usage of telaprevir (Charlton, 2011) but proactive clinicians faced with little other option have reported firstly single centre, on-response treatment data but more recently SVR 12 data. Clinical series have encompassed a wide heterogeneity of patients with one study enrolling approximately one quarter of patients with recurrent cirrhosis and/or re-transplant and/or co-infection with HIV (Coilly A, 2013). To increase tolerability many centres have utilized a PEG/RIB lead in prior to commencement of PI. Several themes emerge: firstly, that dose reduction of immunosuppression (CNIs) is feasible with boceprevir and telaprevir; secondly, that discontinuations rates and tolerability are a significant issue with deaths related to antiviral therapy (sepsis), anaemia, requiring blood transfusion and growth factor support, and other side effects such as renal failure apparent with >25% discontinuation rate; thirdly, that encouraging on treatment response rates translate into only moderately acceptable SVR12 rates of between 48-58% for G1 patients (Coilly A, 2013, Stravitz, 2013, Pungpapong et al., 2013). Whilst this is an improvement the amount of resource and time required to deliver PI based triple therapy in post-LT patients is immense.

Authors	n	AVT regimen	RVR	EVR (%)	EOTR (%)	SVR 12 (%)	Adverse events
Pungpapong et al. 2013	60	BOC (n= 25) TVR (n= 35) - 4 week lead in with PR used in 47%	BOC – 24% TVR – 17%	BOC- NA TVR – 80%	NA	NA	Anaemia Kidney failure (n=23) Acute rejection)n=4) Infection (n=7) Death (n=2)
Coilly et al. 2014	37	BOC (n=18) TVR (n=19) - 4 week lead in with PR used in 70%	BOC – 56% TVR – 47%	BOC – 89% TVR – 58%	BOC – 72% TVR – 40%	BOC – 71% TVR – 20%	Anaemia (92%) Leucopaenia (40%) Kidney failure (n=5) Infection (n=10) Death (n=3) Rash (n=2)

Table 2.5 Experience of use of triple therapy for HCV recurrence post transplantation.

AVT, anti-viral therapy; BOC, boceprevir; EVR, early virologica response; EOTR, end of treatment of response; SVR, sustained virologic response; NA, not available.

Case reports have emerged on the use of daclatasvir (a NS5A complex inhibitor) with PEG/RBV and sofusbuvir (SOF) in patients with FCH and severe HCV recurrence respectively resulting in a SVR(Fontana et al., 2012). Furthermore, a further case report demonstrated PEG-free therapy pre-LT abrogated HCV viral recurrence (Kwo and Tector, 2013). In a phase 2 open label study, patients with HCV received SOF and RBV prior to LT (Curry MP, 2013). Of 39 evaluable patients, 25 (64%) achieved SVR12. The only factor independently associated with likelihood of HCV recurrence was continuous number of days that HCV RNA was not detected using a highly stringent assay (<10 IU/ml) before LT (OR 1.042, 1.012-1.083, p=0.0007). SOF and RBV were well tolerated with no drug-drug interactions post-LT. This early data would suggest a role for SOF and RBV to help reduce recurrence of HCV post-LT.

A prospective, multi-center study (n=40) assessing SOF + RBV for 24 weeks for the treatment of established HCV recurrence post-LT demonstrated no drug-drug

interactions and very few significant side-effects (Charlton MR, 2013). 100% of patients had a rapid virologic response (RVR) and end of treatment response (EOTR) respectively, although final SVR4 rates were only 77%. Potentially, a longer duration of AVT may result in improved SVR rates.

Post transplant, SOF+RBV with or without PEG has been available for compassionate use on an individual case basis for patients with severe HCV recurrence post-LT (Forns X, 2013). In total 115 patients have been approved, with 44 patients having received a SOF containing regimen (32 x SOF +RBV; 12 x SOF + PEG/RBV SVR 4 and 12 rates for the SOF + RBV group were 74% and 60% respectively compared to 56% and 50% in the SOF + PEG/RBV group. 71% of patients demonstrated evidence of re-compensation with resolution of ascites, improvement in encephalopathy, improvement in bilirubin and MELD. Overall, SOF-based therapy was well tolerated in this very sick population with minimal side effects, although 25% patient died due to disease progression, reflecting the true compassionate nature of this patient group. It maybe that AVT with the new DAAs will allow for rescue/salvage therapy in patients whom otherwise would need reconsideration for re-LT. In a study using SOF and low ascending dose RBV for 24 weeks, all patients achieved a RVR and EOT virologic response whilst 70% achieved a SVR (Charlton et al., 2015b). The SVR in patients without cirrhosis was 75% compared to 62.5% in patients with cirrhosis. The SOLAR-1 study assessed ledipasvir, SOF and RBV for 12 or 24 weeks in patients with genotype 1 or 4 HCV with and without cirrhosis post transplantation (Charlton et al., 2015a). Overall reported SVR12 rates were 75%. Patients without cirrhosis had a SVR12 of 96%. SVR 12 rates then varied according to

the degree of severity of cirrhosis (Child-Pugh A 88%, Child-Pugh B 85%, Child-Pugh C 60%). The data demonstrated that 12 weeks was as effective as 24 weeks (Charlton et al., 2015a). The ALLY-1 study was performed in 53 patients with the use of daclatasvir and SOF for 12 weeks (Poordad et al., 2016). The overall SVR 12 rate was 94%. Sub analysis of viral genotype demonstrated a higher SVR12 rate in patients with genotype 1 infection (94%) compared to patients with genotype 3 infection (91%). The French prospective CUPILT study included 130 transplant patients, one third of whom were cirrhotic treated with daclatasvir, SOF with or without RBV for 12 or 24 weeks (Coilly A, 2015). SVR12 rates were 67% and 100% in patients with or without RBV for 12 weeks compared to 96% and 97% in patients with or without RBV in the 24 week arm. These data would suggest RBV is not essential especially renal dysfunction was a common observation (Coilly A, 2015). The use of the Abbvie 3D regimen (paritaprevir/ritonavir, dasabuvir, ombitasvir) with RBV requires adjustments in the CNI dose due to the inhibition of the CYP-3A4 by the ritonavir-boosted paritaprevir (Badri et al., 2015).

8. Hypothesis

The aims of this thesis were to investigate possible non-invasive markers of fibrosis in patients with recurrent HCV post liver transplantation. I plan to determine the role of both recipient and donor IL-28B genotype on HCV recurrence and on response to AVT in the post transplant period. I will also investigate the role of CXCL10 as predictor of fibrosis, and given its role as a marker of ISG activation, alone and combination with IL-28B genotype to predict treatment response to AVT. Finally, I will investigate as to whether miRNAs can help predict HCV recurrence and investigate their role as potential biomarkers for HCV recurrence post transplantation.

The following hypotheses will be tested:

Hypothesis 1: Recipient and donor rs12979860 IL-28B genotype predict the development of fibrosis and HCV recurrence post liver transplantation.

Hypothesis 2: CXCL10 levels predict the development of fibrosis and HCV recurrence post liver transplantation, and in combination with recipient rs12979860 IL-28B genotype help predict response to anti-viral therapy.

Hypothesis 3: miRNA expression profiles from liver grafts can distinguish the severity of HCV recurrence post liver transplantation.

Chapter III

Methods

1. Patients and specimens

Using the prospectively maintained Institute of Liver Studies, King's College Hospital post liver transplant database, all patients that had undergone transplantation for chronic hepatitis C virus infection were identified. This database provides demographic and clinical data pre- transplantation. Key donor data is also provided (age, weight). Data from the post-transplant period includes choice of immunosuppression as well episodes of acute cellular rejection and survival (patient and graft). Although data was available from 1996 – 2011, data was excluded from 1996-2000 due to paucity of clinical data and availability of liver biopsies, DNA, plasma and serum samples. Therefore only data from 2000-2011 was included.

1.1 Liver biopsies

In the immediate post transplant period (0-4 weeks), the need for liver biopsy is dictated by clinical need. Indications for liver biopsy impaired graft function and/or concerns regarding the possibility of acute cellular rejection. Thereafter, protocol liver biopsies are performed at 12 months post liver transplantation and then on an annual basis in all patients that have undergone liver transplantation for HCV at King's College Hospital. Liver tissue specimens are dissected and stored in paraffin before being sliced at 8mm intervals and placed on slides. An adequate sized biopsy was defined as having a minimum length of 2cm with the presence of greater than 11 complete portal tracts.

1.2 Recurrent hepatitis C virus

Recurrence of hepatitis C in the allograft was diagnosed when there was biochemical evidence of allograft hepatitis associated with histological changes consistent with hepatitis due to HCV in the absence of other causes. Biopsies were performed routinely at 7 days and 12 months or in the presence of graft dysfunction in patients with HCV. In non-HCV patients, biopsies were performed at 7 days or in the presence of graft dysfunction. All biopsies in patients with HCV were read by a single pathologist (AQ) who was blinded to the diagnosis of clinical data and scored according to the modified Ishak score with 0 as no fibrosis and 6 as frank cirrhosis (Ishak et al., 1995). For purposes of the studies included in this thesis, biopsies taken at 12 months were graded as demonstrating either slow fibrosis progression ($F<2$) or fast fibrosis progression ($F\geq2$).

1.3 DNA samples

Samples of DNA are extracted from whole blood from recipients and donor at the time of liver transplantation. These are stored at -20^{o}C. Donor and recipient samples were identified by using the HCV post transplant database. DNA concentrations were measured using the Nanodrop (ND1000 spectrophometer) to ensure adequate template concentration prior to amplification. A DNA concentration of 10-20 ng/µl was required. A minimum volume of 6µl was required for each PCR experiment.

1.4 Plasma and serum samples

Plasma and serum samples were stored at -80^{o}C. Plasma samples are routinely taken at the time of HCV viral load quantification. Recipient serum samples are taken at the time of liver transplantation assessment and time of liver transplantation. Donor serum samples are taken at the time of donor organ procurement.

1.5 HCV treatment and outcomes

HCV anti-viral therapy consisted of pegylated interferon (PEG-IFN) α-2a 180mcg/week and weight-based ribavirin (1000mg/day < 75kg or 1200mg/day > 75kg) for 48 weeks irrespective of genotype. A low dose accelerating regimen (LADR) was considered in patients deemed high risk for hepatic decompensation. Treatment outcomes were classified according to EASL guidelines (European Association for the Study of the, 2011). A rapid virological response (RVR) was defined as serum HCV RNA negativity at week 4; an early virological response (EVR) was defined as serum HCV RNA negativity or >2 $\log_{10}$ decline in HCV RNA compared to baseline at week 12; a non responder (NR) was defined as a <2 $\log_{10}$ drop in HCV RNA at week 12 compared to baseline and those with detectable HCV RNA in serum at week 24; and a responder-relapser (RR) was defined as an undetectable HCV RNA at the end of treatment (EOTR) but detectable HCV RNA at 24 weeks post cessation of therapy; a sustained virological response (SVR) was defined as undetectable HCV RNA in serum 24 weeks after treatment cessation.

2. Ethics

The experiments performed in this thesis were in accordance with the Helsinki Principles. As the samples used were stored, archived material, individual consent was not obtained. The study protocols were approved by the Ethics Committee of King's College Hospital, London, United Kingdom before commencement of the experiments.

Chapter VI

Ethics number: 11/NE/0322.

Chapter V

Ethics number: 08/H0704/117

Chapter VI

Ethics number: 05/Q0703/329.

3. rs12979860 IL-28B allele specific polymerase chain reaction

3.1 Agarose gel production

A 2% agarose gel (12 x 24 well) was prepared (8g Agarose in 400ml of TE (Tris-EDTA buffer)). 10`ul of Nancy-520 DNA gel stain was added to the gel, which acted as a fluorescent stain for dsDNA on the agarose electrophoresis gel.

3.2 Primers

For each allele, there is a primer with the variant encoded at the 5' end, and a common primer, at a final concentration of 5uM in the reaction mixture. In addition, each polymerase chain reaction (PCR) contains an invariant DRB primer pair at 2uM, serving as an internal control. Each reaction thus contains 4 individual primers:

Il28B(60)3_T_F GCTCCCCGAAGGCGT
Il28B(60)3_C_F GCTCCCCGAAGGCGC
Il28B (60)3_R CACAATTCCCACCACGAGAC
POS (DRB) F tgccaagtggagcacccaa 0.025 DST
POS (DRB) R gcatcttgctctgtgcagat 0.025 DST

3.3 Chemicals and reagents

3.3.1 Taq polymerase

Supplied at 5U/ul by Qiagen. Stored at -20^{o}C.

3.3.2 PCR mix

1 part 10x buffer (Qiagen), 0.2 part DNTP 100um (Roche), 1.7 part Q solution (Qiagen) + 1 part glycerol (50%) + 0.1 part Mg. This was stored at 4^{o}C for up to a week before being changed.

3.3.3 96 well PCR plates

Thermo-fast, non skirted sterile 96 well PCR plates (Sigma-Aldrich company Ltd, Poole, UK) were used for these experiments.

3.3.4 DNA extraction

Transplant donor and recipient DNA is extracted and stored as per legislative requirements. In 10 cases, DNA was extracted from stored samples by myself. The Qiagen QIAMP DNA Blood Mini Kit was used for DNA extraction.

1. Suspension of spleen cells/blood were brought to room temperature.
2. The heating block was turned on to 56^{o}C.
3. The Equilibrate Buffer AE and distilled water were brought to room temperature.
4. 100µL proteinase K solution was added to 250µL homogenous suspension of blood and mixed well using a 1mL filter tip Pasteur pipette.

5. 250µL Qiagen buffer AL was then added and mixed well, using a 1mL filter tip pipette.
6. The sample was then incubated for 10 minutes at 56^{o} C.
7. The sample was then centrifuged using a microcentrifuge to remove drops from the inside lid.
8. For each sample, a spin column, 4 collection tubes and a 1.5mL specimen tube was labeled.
9. 200µL of ethanol (100%) was added to the sample and mixed well with a filter tipped pipette.
10. The samples were then transferred to spin columns, ensuring not to wet the rims.
11. The samples were then centrifuged (microcentrifuge) at 13000 rpm for 1 minute,
12. The collection tube containing the filtrate was then discarded into a yellow incineration bin lined with appropriate absorbent material for disposal.
13. AW1 and AW2 buffers were then decanted into clean 20mL tubes.
14. 500µL buffer AW1 was then added to each column.
15. Samples were then centrifuged at 13000 rpm for 1 minutes. The collection tube containing the filtrate was then discarded into a yellow incineration bin lined with appropriate absorbent material for disposal.
16. 500µL buffer AW2 was then added to each column.

17. Samples were then centrifuged at 13000 rpm for 1 minute. The collection tube containing the filtrate was then discarded into a yellow incineration bin lined with appropriate absorbent material for disposal.

18. Samples were then centrifuged at 13000 rpm for 2 minutes. The collection tube containing the filtrate was then discarded into a yellow incineration bin lined with appropriate absorbent material for disposal.
19. The spin column was then transferred to a clean labeled 1.5mL collection tube (not a microcentrifuge tube).
20. 100µL buffer *AE* was added to the column.
21. Samples were then Incubated at 55^{o} C for 10minutes.
22. Samples were then centrifuged at 13000 rpm for 1 minute.
23. Samples from the collection tube were then transferred to a clean labeled screw-top tube, checking the sample ID .
24. 500µL of buffer AE was added to the column.
25. Samples were then incubated at 55^{o} C for 10minutes.
26. Samples were then centrifuged at 13000 rpm for 1 minute.
27. The spin column was then discarded and the sample was transferred from the collection tube to a clean labeled screw-top tube, checking the sample ID
28. The concentration and purity of each DNA extraction was calculated using the Nanodrop.

3.4 Preparing samples for genotyping

The IL-28B PCR was performed with a volume of 20μl, containing 14μl of 'mastermix' and 6ul of DNA template.

1. DNA samples were defrosted on ice and brought to room temperature. 48 DNA samples were run at a time.
2. A work sheet was labelled with the samples that were going to be used.
3. A sterile 96 well plate was placed on ice.
4. 8ul of cocktail, 0.2μl of Taq polymerase and 6μl of primer was prepared for each sample in an Eppendorf tube e.g. for 60 samples, 480ul cocktail, 12μl Taq polymerase and 360μl primer. Samples were then vortexed. 14μl of the reaction mix was then added to each reaction well for each sample.
5. Next, 6μl of DNA was added to each reaction well ensuring the samples were mixed.
6. The PCR plate was then covered with a PCR plate sealer, ensuring that all wells were covered.
7. A negative control (14μl reaction mix + 6ul water) was included on each plate.

3.5 PCR reaction

Touch down PCR:
94^{o} 60s
94^{o} 15s
70^{o} 60s (2 degrees/cycle decrement)
(5 cycles)

94^{o} 15s
60^{o} 30s (0.5 degrees /cycle decrement)
72^{o} 45s
(16 cycles)

94^{o} 15s
52^{o} 30s
72^{o} 60s
(12 cycles)

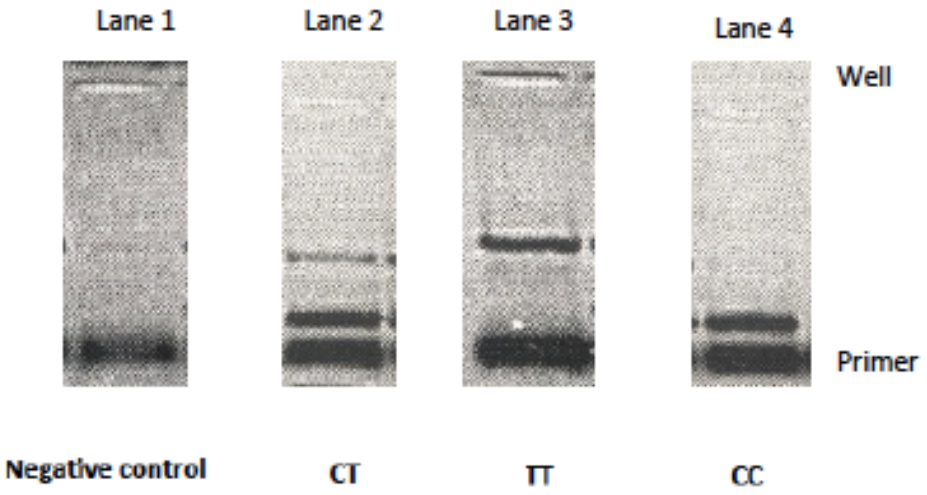
3.6 Agarose gel electrophoresis

An 8-channel Pasteur pipette was used to load 20μl of sample from the PCR plate onto the gel and then run at 250V (Hybaid PS250, Tank supplied by Alpha Laboratories) at maximum amps for 20 minutes.

3.7 Interpretation of results

For each polymorphism (IL-28B), two PCR's were performed. At least one band must be visible in each PCR for a result to be valid. If no band is visible in either PCR, the result was repeated or discounted. A positive band corresponding the respective alleles could only be interpreted as homozygous if the internal control band for the other allele was clearly present and the allele specific band was absent. My results were validated by James Underhill.

Figure 3.1 Agarose gel electrophoresis photograph pf the PCR products obtained with the IL-28B forward and reverse primers. First lane demonstrates the negative control. Lane 2 demonstrates amplification of the CT band, lane 3 TT band and lane 4 CC band.



4. CXCL10 quantification

The commercially available R&D systems ELISA Quantikine kit was used.

4.1 Reagent preparation

All reagents were brought to room temperature. Plasma samples were defrosted on ice. 20ml of wash buffer concentrate was diluted into 500mls of deionised water. Colour reagents A and B were mixed together within 15 minutes of use. 200μL of the resultant mixture was added to each well. The CXCL10 Standard was reconstituted with 1.0ml of deionised water. A resultant stock solution of 5000pg/ml was produced which was allowed to sit for 15 minutes with gentle agitation. Seven polypropylene tubes were taken and labelled 500pg/mL, 250mg/mL, 125pg/mL, 62.5pg/mL, 31.2pg/mL, 15.6pg/mL and 7.8pg/mL 900uL of calibrator diluent RD6Q was then pipetted into the tube labelled 500pg/ml. 500uL of calibrator diluent RD6Q was then subsequently pipetted into the remaining tubes. 100uL of the stock solution was then pipetted into the 500pg/mL tube before a serial dilution was performed taking 500uL from each tube and placing it into the next subsequent tube. The 500pg/mL standard served as the high standard whilst the calibrator diluent RD6Q served as zero standard (0 pg/mL).

4.2 Assay procedure

1. 75μL of assay diluent RD1-56 was added to each well.
2. 75μL of Standard and then 75μL of plasma was added to each well. Patient samples were performed in triplicate. A mean value of the triplicates were used for analysis. The plate was then covered and incubated for 2 hours at room temperature.
3. Each well was then aspirated and then washed using 400μL of wash buffer. Complete removal was performed. This process was repeated 3 times for a total of 4 washes. After the last wash any remaining fluid was removed by balloting the plate.
4. 200μL of CXCL10 conjugate was then added to each well. The plate was covered and then incubated for a further 2 hours at room temperature. Each well was then aspirated and washed as explained above.
5. 200μL of substrate solution was then added to each well before being incubated for 30 minutes at room temperature. Direct exposure to light was avoided.
6. 50μL of stop solution was then added to each well. The colour of the solution in the wells changed from blue to yellow. To ensure that adequate mixing had occurred, the plate was gently tapped.
7. Within 30 minutes, the optical density was determined for each well using a microplate reader set to 450 nm.

5. Isolation of RNA from formalin-fixed paraffin-embedded tissue

5.1 Deparaffinisation

1. 10μm paraffin embedded liver tissue sections were selected. Sections were placed in a 1.5ml reaction tube. 800μL of xylene was added and vortexed for 4 seconds (3 times). Samples were then incubated for 2 minutes at room temperature (RT), vortexed for 4 seconds (3 times) and then incubated for 5 minutes at RT. Samples were then centrifuged for 2 minutes (13000rpm). The supernatant was discarded by aspiration and tubes capped to avoid tissue sections from drying.
2. Step 1. was then repeated.
3. 800uL of absolute ethanol (100%) was then added to each reaction tube and then vortexed for 4 seconds (3 times). Reaction tubes were then centrifuged for 2 minutes (13,000 x g). The supernatant was discarded by aspiration.
4. 800ul of 70% ethanol was then added to each reaction tube and vortexed for 4 seconds (3 times). Samples were then centrifuged for 2 minutes (13,000 x g). The supernatant was then discarded by aspiration.
5. 70% ethanol was removed and the reaction tubes were centrifuged for 20 seconds and the residual fluid carefully removed with a fine bore pipette. The reaction tubes were opened and incubated for 5 minutes at 55^{o} C (in a waterbath) in order to dry the tissue pellet.

5.2 RNA isolation from liver tissue sections

1. 60μL of tissue lysis buffer and 10uL 10% SDS (sodium dodecyl sulphate) was added to each deparaffinised tissue pellet (sample). Each sample was then vortexed for 4 seconds (3 times) and centrifuged (13,000 rpm). Then 30μL Proteinase K working solution was added and vortexed for 4 seconds (3 times), centrifuged and then incubated for 12 hours at 55^{o} C in a water bath.
2. 200μL of binding buffer and 200μL absolute ethanol (100%) was added to each sample and then vortexed for 4 seconds (3 times).
3. The high pure filter microtube (HPMT) was combined with a collection tube and each lysate was individually pippetted into the upper reservoir and then centrifuged for 30 seconds (8,000 rpm) in a micro centrifuge. The flow-through was then discarded.
4. The samples were then centrifuged for 1 minute at maximum speed to dry the filter.
5. 30μL DNase solution (3μL DNase Incubation Buffer and 27μL DNase) was then added and the samples were incubated for 15 minutes at RT.
6. 300μL Wash Buffer I working solution was added to the upper reservoir and centrifuged for 15 seconds (8,000 rpm). The follow through was then discarded and 300μL Wash Buffer II working solution was added to the upper reservoir and then centrifuged for 15 seconds (8,000 rpm). The follow through was then discarded.

7. Again 200μL Wash Buffer II working solution was added to the upper reservoir and centrifuged for 15 seconds (8,000 rpm). The follow through was then discarded.
8. The HPMT tubes were then placed in a fresh collection tube and then centrifuged for 2 minutes at maximum speed and then HPMT tube was placed in a fresh 1.5ml reaction tube and 20μL Elution Buffer was added. Samples were then incubated for 1 minute at room temperature. Samples were then centrifuged for 1 minute (8,000 rpm).
9. The eluate was reloaded (contents of the tube); incubated for 1 additional minute at RT and then centrifuged for 1 minute (8,000 rpm).
10. The micro-centrifuge tube contained the eluted RNA. Samples were then centrifuged for 2 minutes at 13,000g and the supernatant was transferred to a fresh 1.5ml reaction tube without disturbing the glass fibres at the bottom of the original tube before photometric determination of the RNA concentration.
11. Then 1.2μL of each sample was checked for purity (ratio of 260 to 280 nm) and concentration (ng/μL) using the nano-drop spectrometer.

5.3 De-paraffinisation and RNA isolation experiments using normal liver tissue

Using the methods described in section 5.1 and 5.2, normal liver tissue was taken and used in order to ensure an adequate yield of RNA. 6 samples were used. The results are listed below demonstrating suitable concentrations and purity. A minimum of 1ng per sample of total RNA was required. The ratio of absorbance at 260nm and 280 nm was used to assess the purity.

Sample Number	Concentration (ng/μL)	Purity (260nm/280nm)
1	3.0	1.39
2	88.9	2.11
3	60.3	2.14
4	34.2	1.38
5	30.4	1.42
6	86.1	1.87

Table 3.1 Concentration and purity of RNA yield in normal liver tissue.

5.4 De-paraffinisation and RNA isolation from study samples

Study samples used, were stored samples and although adequate RNA yields are described in the literature we wanted to ensure an adequate yield was possible with our samples. Using the methods described in 5.1 and 5.3, samples from 6 patients were processed initially. The results are listed below of the concentrations and purity (Table 3.2). Finally, the methods were repeated for the study cohort (Table 3.3).

Sample Number	Concentration (ng/μL)	Purity (260nm/280nm)
1	5.3	6.0
2	15.1	2.21
3	8.2	1.43
4	15.0	1.78
5	3.8	1.63
6	19.1	1.97

Table 3.2 Concentration and purity of RNA in preliminary study samples.

Sample Number	Concentration (ng/uL)	Purity (260nm/280nm)
Group A		
1	5.0	6.41
2	15.4	2.33
3	8.2	1.43
4	38.6	2.12
5	15.7	1.81
6	4.4	2.0
7	5.0	6.41
8	15.4	2.33
9	8.2	1.43
10	38.6	2.12
11	15.7	1.81

Sample Number	Concentration (ng/uL)	Purity (260nm/280nm)
Group B		
1	19.1	1.97
2	5.5	1.70
3	20.8	2.07
4	8.3	0.76
5	19.1	1.97
6	6.9	0.9
7	5.5	1.7
8	20.8	2.07
9	3.8	1.55

Sample Number	Concentration (ng/uL)	Purity (260nm/280nm)
Group C		
1	14.9	2.03
2	16.1	1.98
3	7.5	1.83
4	15.0	1.78
5	52.5	1.87

Sample Number	Concentration (ng/uL)	Purity (260nm/280nm)
Group D		
1	5.8	2.08
2	12.9	1.91
3	1.3	1.93
4	3.8	1.5

Table 3.3 Concentration and purity of RNA in study samples.

6. FlashTag RNA labelling

FlashTag labelling allows for the RNA to be used for analysis by Affymetrix GeneChips miRNA Arrays. The process involves 2 parts: tailing followed by ligation of the biotinylated signal molecule to the target RNA sample.

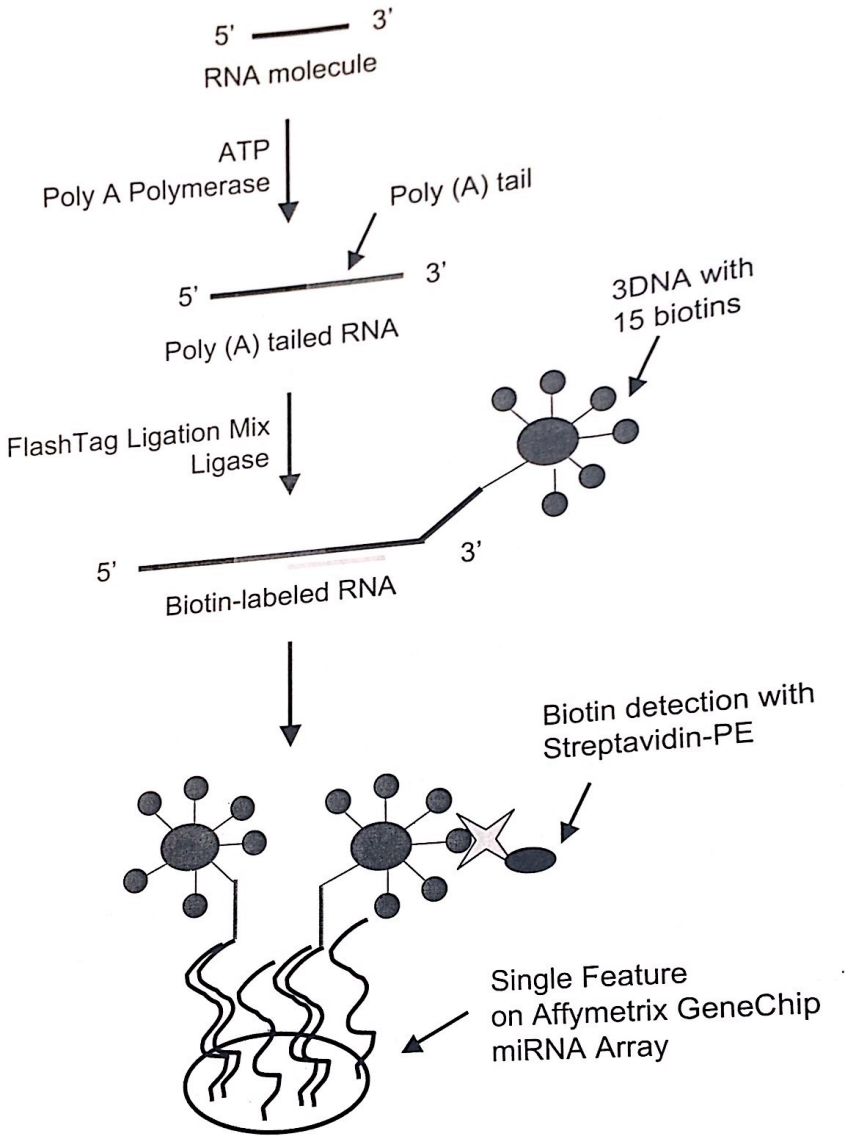


Figure 3.2 FlagTag RNA labelling
(adapted from Affymetrix RNA labelling kit protocol).

6.1 Poly (A) Tailing

1. The volume of RNA was adjusted to 8μL using nuclease free water and then transferred to ice. 2μL of RNA Spike Control Oligos (Genisphere Inc. Hatfield) was added to each sample before returning to ice (total volume 10 μL).
2. The ATP mix was diluted to a 1:500 using 1mM Tris and the following components were added to the 10μl RNA/Spike Control Oligos, for a volume of 15μl:
    - 1.5 μl 10X reaction buffer
    - 1.5 μl 25mM $MnCl_2$
    - 1.0 μl diluted ATP Mix
    - 1.0 μl PAP enzyme
3. Samples were then mixed and microfuged and incubated at 37^{o}C using a heat block for 15 minutes.

6.2 FlashTag ligation

1. Samples containing 15μl of the tailed RNA were microfuged and then placed on ice.
2. 4 μl 5X flashTag ligatation mix Biotin (Genisphere Inc. Hatfield) was added to each sample followed by the addition of 2μl T4 DNA ligase.
3. Samples were mixed gently but not vortexed and then incubated at RT for 30 minutes.
4. The reaction was stopped by adding 2.5μl Stop solution and the final volume of the 23.5μl ligated sample was mixed and then microfuged.
5. Samples were then stored at -20^{o}C for up to 2 weeks prior to hybridization on Affymetrix GeneChip miRNA arrays (Genisphere Inc. Hatfield).

7. Affymetrix GeneChip miRNA Array procedure

7.1 Hybridization

Experiments were performed at the Genomics Centre, King's College London, Waterloo Campus, London, UK.

1. Affymetrix hybridisation (Genisphere Inc. Hatfield) oven's temperature was set to 48^{o}C. The rpm was set to 60.
2. The arrays were unwrapped and allowed to warm to room temperature for 15 minutes. The arrays were marked with the appropriate sample number.
3. A 20μl pipette tip was inserted into the upper right septum to allow venting when the hybridization cocktail was injected.
4. The following components were added (in the order listed) to each of the 21.5μl biotin-labelled sample in order to prepare the array hybridisation cocktail:
    - 50 μl 2X hybridisation mix (GeneChip ® Hybridization, wash, and stain kit; Affymetrix)
    - 10 μl nuclease free water
    - 5μl deionised formamide, molecular biology grade
    - 10 μl DMSO (dimethyl sulphoxide)
    - 5 μl 20X eukaryocyte hybridisation controls (GeneChip ® Hybridization Control kit, Affymetrix)
    - 1.7 μl control oligonucleotide B2, 3nM (GeneChip ® Hybridization Control kit, Affymetrix)
5. The total volume of the sample was 103.2μl which was then incubated at 99^{o}C for 5 minutes and then centrifuged before incubated at 45^{o}C for 5 minutes.

6. 100 μl was aspirated and then injected into the array.
7. The 20μl pippette was removed from the upper right septum of the array.
8. Both septa were then covered to minimize evaporation and/or leaks
9. The arrays were placed into the hybridization oven trays and incubated for 48^{o}C and 60rpm for 16 hours. Samples were loaded at approximately 1700 and left over night.

7.2 Washing and staining

1. After the 16 hours of hybridization, the arrays were removed from the oven.
2. The hybridization cocktail was removed from each array and transferred into a new Eppendorf microtube (Hamburg, Germany). Samples were then stored at -80^{o}C.
3. 120 μl of the Array holding buffer was then inserted into each array.
4. The arrays were checked for bubbles and dust. Both septa were covered to minimize leakage. The array glass surface was also inspected for dust and cleaned appropriately.
5. The fluidics station was prepared appropriately with the following:
    - 600 μl of Stain Cocktail 1 was inserted into sample holder 1
    - 600 μl of Stain Cocktail 2 was inserted into sample holder 2
    - 800 μl of Array Holding buffer was inserted in sample holder 3
6. The fluidics station was washed and stained as below:

Post Hyb wash # 1	10 cycles of 2 mixes/cycle with Wash Buffer A at 25^{o}C
Post HyB Wash # 2	8 cycles of 15 mixes/cycle with Wah Buffer B at 50^{o}C
1st Stain	Stain the probe array for 10 minutes with Stain cocktail 1 at 25^{o}C

Post Stain wash	10 cycles of 4 mixes/cycle with wash Buffer A at 30°C
2nd Stain	Stain the probe array for 10 minutes with Stain cocktail 2 at 25°C
3rd Stain	Stain the probe array for 10 minutes with Stain cocktail 1 at 25°C
Final wash	15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C
Array holding buffer	Fill the probe array with Array holding buffer

7.3 Scanning

Scanning was performed using the Affymetrix command console software. The software is able to summarise probe cell intensity data (CEL file generation) and enables sample and array registration, data management, instrument control as well as automatic and manual image gridding.

7.4 GeneChip miRNA 2.0 Arrays

GeneChip miRNA 2.0 arrays (Affumetrix) were used.

7.5 Analysis of scanned miRNA arrays

The array images (CEL files) were processed using the Affymetrix's miRNA QC Tool, applying the default workflow, which includes background correction summarisation of multiple probes into overall probeset intensity and normalistation based on Quantile normalistation. The procedure generates log 2 intensities for the 15,644 probe sets on the chips for each sample. The microarray data is MIAME (minimum information about a microarray experiment) compliant and has been deposited in the GEO repository (Accession no. GSE40113).

8.miRNA analysis

8.1 Qlucore Omics explorer

The Qlucore omics explorer software at the Genomic Centre, King's College London, Waterloo Campus, London, UK was used. Data was exported from the miRNA QC Tool and formatted as a 'gedata' tab –delimited text file and imported into Qlucore's Omics Explorer 2.1 software for analysis. A simple 1-way ANOVA (analysis of variance) was used to filter genes that were differentially regulated between the different groups. P values were set to 0.01. Data was checked for outliers by visual inspection using principal component analysis (PCA) plots. Gene lists containing all regulated genes were displayed as heatmaps to show gene expression patterns.

8.2 Metacore analysis

Metacore is an integrated web-based software suite for functional analysis of microarray, metabolic, proteomics and miRNA data. It is based on a high quality, manually curated database of transcription factors, receptors, ligands, kinases, drugs and endogenous metabolites. It also provides data on signalling and metabolic pathways represented on maps and networks. Gene lists identified were exported as excel files. The enrichment analysis performed by Metacore examines the intersection between one's data and prebuilt pathway maps and networks in Metacore. Networks are then created providing a p-value, Z-score and G-score. The p-value represent the probability of these networks occurring purely by chance. The Z-score calculates the level of saturation of the networks taking into account the size of the database, the number of objects in the sub-network and the number of objects in the data use to

construct the network. The higher the Z-score the more saturated a sub-network is with ones data. The G-score modifies the Z score where possible based on the number of the linear canonical pathway fragments contained within the network. The genetic pathways known to be regulated by these miRNAs were therefore interrogated.

9. RNA purification from serum

RNA was extracted from serum according to Qiagen's miRNeasy mini kit protocol.

9.1 Materials

- miRNeasy mini kit (50) (Qiagen).
- Extra RPE-buffer, (Qiagen)
- MS2 RNA (0.8 µg/µl) (Roche)
- Chloroform (Sigma Aldrich)
- Absolute ethanol (99.9%)
- RNase-free filtertips
- RNase-free 1.5 ml and 2 ml tubes, (e.g. Non-Stick RNase-free Microfuge Tubes) (Ambicon)
- Table-top micro centrifuge with cooling, capable of 13,000 rpm centrifugal force

9.2 Protocol

1. Serum and MS2 RNA were thawed on ice. Samples were kept on ice.
2. The centrifuge was cooled to 4°C.
3. 250 µl of serum was placed into a 1.5 ml tube. And centrifuged at 3,000 rpm for 5 minutes at 4°C to remove debris and then 200 µl of serum was transferred to a new 1.5 ml tube.

4. QIAzol master mix (800 µl QIAzol + 1.25 µl MS2) was made per sample and vortexd briefly to mix.
5. 750µl QIAzol master mix was then added to each 200 µl serum sample and vortexed.
6. The samples were incubated for 5 minutes at room temperature and then 200µl chloroform was added to each sample and vortexed.
7. Samples were then incubated for 2 minutes at room temperature and then centrifuged at 12,000 rpm for 15 minutes at 4°C.
8. The centrifuge was then heated to room temperature (20°C) and the upper aqueous phase was transferred to a 2 ml tube.
9. 1.5 volumes (525 µl) of 100% ethanol were added and mixed by pipetting. 750µl of the solution was transferred to a RNeasy mini spin column and carefully marked.
10. Samples were centrifuged for 30 sec at room tempertaure (13,000 rpm). The flow-through was discarded.
11. Step 10 was repeated until all of the sample was used (samples were remixed by pipetting before loading again).
12. 700 µl RWT wash solution was added and centrifuged for 1 min at room temperature (13,000 rpm). The flow-through was then discarded.
13. 500µl RPE was added to wash the samples and repeated 3 times. After each addition samples were centrifuged for 1 min at room temperature (13,000 rpm) and flow-through discarded.
14. Columns were then transferred to a new, labelled collection tube and centrifuged for 2 minutes at room temperature (13000 rpm).

15. The tubes were left open for 1 min and columns were transferred to new non-stick RNase-free Microfuge. Then 50µl DNase/RNase-free water was added, making sure the liquid was centered on the membrane and incubated for 1 min and then centrifuged for 1 min at room temperature, (13,000 rpm).
16. The final RNA solution obtained was stored at -80°C.

10. cDNA synthesis

For each RNA sample, the concentration was adjusted to 5ng/µl using nuclease free water. The reagents were prepared. The 5x Reaction buffer (Exiqon Inc.) and nuclease free water were gently thawed by placing them on ice before being vortexed and mixed. The RNA spike was re-suspended by adding in 40 µl of nuclease free water to the tube before vortexing prior to use (left on ice for 15-20 minutes). Immediately before use, the enzyme mix was removed from the freezer by flicking the tubes. All agents were then centrifuged. Reagent volumes are listed below.

Reagent	Volume (μl), RT reaction
5x Reaction buffer	4
Nuclease free water	10
Enzyme mix	2
Template total RNA (5ng/μl)	4

Table 3.4 Reverse transcription (RT) reaction setup

The RT master mix was prepared and placed on ice before being dispensed (16μl) into nuclease free tubes. The samples were gently vortexed and then centrifuged. Samples were incubated for 60 minutes at 42^{o}C.The RT was then heat inactivated by placing the samples at 95^{o}C for 5 minutes. Samples were then cooled to 4^{o}C and stored at 4^{o}C.

11. Real-time quantitative PCR (qPCR)

Selected miRNAs were analysed using liver tissue and serum using the miRCURY TM LNA Universal RT microRNA PCR system (Exiqon).

Primers were re-suspended and mixed by adding 110 μl nuclease free water to each tube. Tubes were then vortexed and centrifuged down before being left on ice for 30 minutes. The PCR primer was prepared by adding 110 μl of the Fwd (forward) primer to 110 μl of the Rev (reverse) primer. Before using the reference gene primer for the first time, the primers were re-suspended by adding 220 μl of nuclease free water to

each tube. All tubes were vortexed and centrifuged before then being left on ice for 30 minutes. Negative controls of nuclease free water were also used.

11.1 Real-time PCR amplification

1. The prepared cDNA, nuclease free water and SYBR® Green master mix were placed on ice and thawed for 20 minutes. SYBR Green master mix is light sensitive and was therefore protected from light by using a cover. Immediately before use, the SYBR® Green Master was mixed by pipetting up and down.
2. 2.5 μl of cDNA was taken and added to 197.5 μl of nuclease free water to achieve a 1:80 dilution. 'Low-nucleic acid binding' tubes were used. Rox dye was used as a passive reference dye in the cDNA solution.
3. A PCR master mix of the PCR primers and SYBR® Green master mix (QiIAGEN) was made as follows:

Reagent	Volume (μl), 384 well plate
SYBR® Green Master mix	5
PCR primer mix	1
Rox dye	0.2
Diluted cDNA template	4

Table 3.5 Real-time PCR reaction, for a 10ul reaction

4. The reaction was then mixed gently by pipetting to ensure all the reagents are thoroughly mixed and centrifuged (15,000 rpm for 1 minute).

5. Real-time PCR amplification was performed followed by melting curve analysis with the following settings:

- Polymerase Activation/ Denaturation	95˚C, 10 min
- Amplification	40 amplification cycles at 95˚C, 10 s, 60˚C, 1 min, ramp-rate 1.6˚C/s7) Optical read
- Melting curve analysis	Yes

6. Samples were run in triplicate.

12. Statistical analysis

Continuous variable were expressed as medians and ranges (minimum and maximum). Comparison was made using non-parametric tests. Categorical variables were compared using the Chi-square, Mann-Whitney U test or Fisher's Exact test. A 2-tailed P-value < 0.05 was considered to indicate statistical significance. Analysis was performed by myself using SPSS for Windows 18 (SPSS, Chicago, IL, USA).

Chapter IV

The independent effects of recipient IL-28B genotype, donor age and diabetes mellitus on histological recurrence (F≥2 at 12 months) were determined using logistic regression analysis. Recipient IL-28B genotype, diabetes mellitus, donor age, recipient age, SVR and HCV viral genotype were included in a Cox-regression model to help predict F≥4 post transplant.

Chapter V

Correlation coefficients of CXCL10 with aspartate transaminase (AST), alanine transaminase (ALT), necro-inflammatory (NI) score and fibrosis (F) score using the Spearman rank correlation. The ability of variables to predict F≥2 at 12 months was assessed using the area under the curve generated by receiver operator characteristic analysis. The area under the receiver operating curve (AUROC) was used to assess the ability to distinguish categorical state. Receiver-operator characteristic (ROC) curves were compared using the Hanley McNeil method (Hanley and McNeil, 1983). The point of highest sensitivity and specificity was defined using the Youden method (Youden, 1950). The independent effects of CXCL10 levels, donor age and diabetes mellitus on histological recurrence (F≥2 at 12 months) were determined using logistic regression analysis. CXCL10, diabetes mellitus, donor age, recipient age and HCV viral genotype were included in a Cox-regression model to help predict F≥4 post transplant. The independent effects of HCV genotype, CXCL10 levels, recipient age, IL-28B genotype and advanced fibrosis (F>4) on treatment outcomes were determined using logistic regression analysis.

Chapter VI

For qPCR statistical analysis, data was expressed as mean+/- standard deviation. The Student's t-test was used to determine statistical difference between group A and B.

Chapter IV

The role of donor and recipient rs12979860 IL-28B polymorphisms in HCV recurrence post liver transplantation

1. Background

Large epidemiological studies have identified a number of candidate genes that influence fibrosis (Huang et al., 2007). Single nucleotide polymorphisms (SNP) in the interleukin (IL)-28B gene located on chromosome 19, rs12979860, have been demonstrated to strongly influence HCV clearance and treatment response (Ge et al., 2009). In particular post transplant, recipient IL-28B TT genotype was associated with a more rapid fibrosis progression rate (Lange and Zeuzem, 2011, Coto-Llerena et al., 2011, Eurich et al., 2011). The role of donor IL-28B genotype appears to be less established with regards to fibrosis progression with conflicting data. There is no published data regarding the role of IL-28B genotype and fibrosis rates post transplantation from the UK. I therefore, aimed to examine the role of rs12979860 IL-28B polymorphisms in both donor and recipient DNA on outcomes in patients with HCV recurrence post-transplantation. Institutionally, performing the IL-28B genotyping on recipient and donor DNA was important step in order to characterise our large cohort of patients.

2. Subjects

Between January 2000 and January 2011, 303 adult patients (> 18 years) with chronic HCV underwent primary liver transplantation at King's College Hospital NHS Foundation Trust, London, UK. Patients were identified using the Institute of Liver Studies viral hepatitis computerized database. Patients with a fibrosing cholestatic hepatitis (FCH) (n=5), those undergoing re-transplantation (n=13) were excluded along with individuals co-infected with human immunodeficiency virus (HIV, n = 8), chronic hepatitis B virus (HBV, n = 12) and those undergoing joint liver-kidney transplantation

(n=1). Patients that did not survive a minimum of 12 months were also excluded (n=74). 57 patients did not have a liver biopsy performed at 12 months as they were not followed up at our institution. Of the remaining patients, 133 had a 12 month liver biopsy performed. Median number of biopsies per patient was 2 (range 1-4). Patients were considered for AVT once there was evidence of progressive fibrosis (F≥2, Ishak) on liver biopsy. Institutional ethical approval was obtained prior to commencement of the study.

3. Results

3.1 Patient characteristics

303 patients (82% male) underwent liver primary liver transplantation during the study period. All patients had virological recurrence of HCV infection post transplant. Patient demographics are listed in Table 4.1. 236 patients survived for greater than 12 months. 133 patients were included in the final analysis. Median recipient age was 53 years (range 19-67 years) and median donor age was 46 years (range 15-87 years). Tacrolimus based immunosuppression was used in the majority of patients (98%).

3.2 Donor & recipient rs12979860 IL-28B genotype

Donor and recipient rs12979860 IL-28B genotyping was performed successfully in 63 and 126 patients respectively (Table 4.2 and 4.3). The IL-28B CT genotype was the most prevalent amongst donors and recipient, 44% and 48% respectively. Paired donor and recipient samples were available in 59 patients. The frequencies were: donor non-

CC/recipient non-CC = 24% (n=14), donor CC/recipient non-CC = 34% (n=20), donor non-CC/recipient CC = 32% (=19), donor CC/recipient CC = 10% (n=6).

	N=133
Recipient Age (years)	53, 19-67
Male (n/%)	110/82
Caucasian (n/%)	109/81
BMI (kg/m^2)	26, 18-41
HBcAb (n/%)	31/23
ALD (n/%)	23/23
HCV genotype 1 (n/%)	73/55
HCC (n/%)	51/38
Diabetes (n/%)	73/55
Donor age (years)*	46, 15-87
DRI	1.7, 1.1-3.1
DBD (n/%)	90/68
CIT (hours)	10.8, 1.6-19.0
Tacrolimus (n/%)	130/98
MELD	13, 5-40
CPS	9, 5-14
Patient Survival (m)	35, 1-145
Time to F≥2 (m)	22, 3-124
Time to F≥4 (m)	34, 5-124

Table 4.1 Patient demographics and characteristics at the time of liver transplantation.

BMI, body mass index; ALD, alcohol induced liver disease; HCV, hepatic C virus; HCC, hepatocellular carcinoma; DRI, donor risk index; DBD, donation after brain death; CIT, cold-ischaemia time; MELD, model for end stage liver disease; CPS, Child Pugh score; m, months. *. * Number of donors included was limited to the number of patients with valid donor DNA (n=63).

Genotype SNP	HCV patients post transplant
IL-28B (n)	63
TT (n/%)	8/13
CT (n/%)	28/44
CC (n/%)	27/43
T allele frequency (%)	70%
C allele frequency (%)	87

Table 4.2 Donor rs12979860 IL-28B genotype frequency.

Genotype SNP	HCV patients post transplant
IL-28B (n)	126
TT (n/%)	20/16
CT (n/%)	60/48
CC (n/%)	46/36
T allele frequency(%)	80
C allele frequency(%)	84

Table 4.3 Recipient rs12979860 IL-28B genotype frequency.

3.3 Patient and graft survival

No difference in patient (83% vs. 71%, p =0.2) or graft (81% vs. 67%, p=0.2) survival was observed according to recipient IL-28B genotype (CC vs. non-CC) at 5 years. Patient (93% vs. 82%, p=0.3) and graft (82% vs. 68%, p=0.2) survival did not differ statistically according to donor IL-28B genotype (CC vs. non-CC) at 5 years.

3.4 Fibrosis progression

Recipient non-CC rs12979860 IL-28B genotype was more likely to be associated with a rapid fibrosis progression rate (F≥2 at 12 months); 26% vs. 41%, p=0.05. Multivariate regression identified recipient rs12979860 IL-28B non-CC genotype to be predictive of F≥2 at 12 months (Table 4.4). The number of donor IL28B samples that were available were small and so it was not included in the model to help predict F≥2 at 12 months or F≥4. The recipients with the CC IL-28B genotype were less likely to develop F≥4 post transplant compared to patients with non-CC genotype (11% vs. 48%, p<0.0001). Progression to F≥4 was also attenuated at 5 years (19% vs 56%, p=0.004) amongst recipient CC IL2-8B patients. On multivariate regression analysis, recipient rs12979860 IL-28B CC genotype vs. non-CC genotype was predictive of progression to F≥4 (OR 0.4, 0.04-0.45, p=0.007) along with donor age and diabetes mellitus (Table 4.5). The donor CC IL-28B genotype was not statistically associated with F>2 at 12 months (35% vs. 38%, p=0.7) but were less likely to develop F≥4 in the post transplant period (48% vs. 83%, p=0.03). Patients with the donor/recipient 12979860 IL-28B CC combination were less likely to develop rapid fibrosis compared to the donor/recipient non-CC rs12979860 IL-28B genotype (24% vs. 53%, p=0.05). Donor /recipient IL-28B CC genotype patients were also less likely to develop F≥4 (11% vs. 78%, p<0.0001).

Parameter	Odds ratio	95% CI	P value
Univariate			
Recipient age	0.9	0.9-1.09	0.1
Donor age	1.9	0.68-5.6	*0.2*
Recipient IL28B CC vs non CC	0.3	0.13-0.67	*0.003*
DM	1.6	0.82-2.9	*0.17*
MELD	0.9	0.92-1.03	*0.4*
Multivariate			
Recipient age	0.9	0.9-1.02	*0.24*
Recipient IL28B CC vs non CC	0.28	0.13-0.66	*0.03*

Table 4.4 Factors predictive of rapid fibrosis progression (F≥2 at 12 months).

Parameter	Odds ratio	95% CI	P value
Univariate			
Recipient age	0.96	0.93-0.99	0.03
Donor age	6.3	2.3-17.1	*<0.0001*
MELD	1.04	0.98-1.11	*0.13*
Recipient IL28B CC vs non CC	0.2	0.04-0.42	*<0.0001*
Diabetes	2.1	0.9-3.6	*0.05*
SVR	0.43	0.03-0.77	*0.001*
Multivariate			
Recipient Age	0.9	0.84-1.02	*0.1*
Donor age	3.3	2.7-15.4	*0.04*
Diabetes	2.9	0.1- 2.27	*0.05*
Recipient IL28B CC vs non CC	0.4	0.04-0.45	*0.007*
SVR	0.38	0.02-0.72	*0.01*

Table 4.5 Factors predictive of F≥4 post transplant.

3.5 Treatment response

56 of the 59 patients that had donor and recipient IL-28B genotypes available had been exposed to AVT, the majority (89%) with PEG/RBV. Pre-transplant treatment data was only available in 7 patients (3 responder relapsers, 2 non responders and 2 patients intolerant of AVT). Median time to treatment was 24 months (range 12-68 months). SVR rates according to donor/recipient combination are shown in Figure 4.1. Donor and recipient IL-28B CC genotype had 100% SVR rates (n=6) in comparison to SVR rates of 25% amongst donor and recipient IL-28B non-CC genotype (n=12).

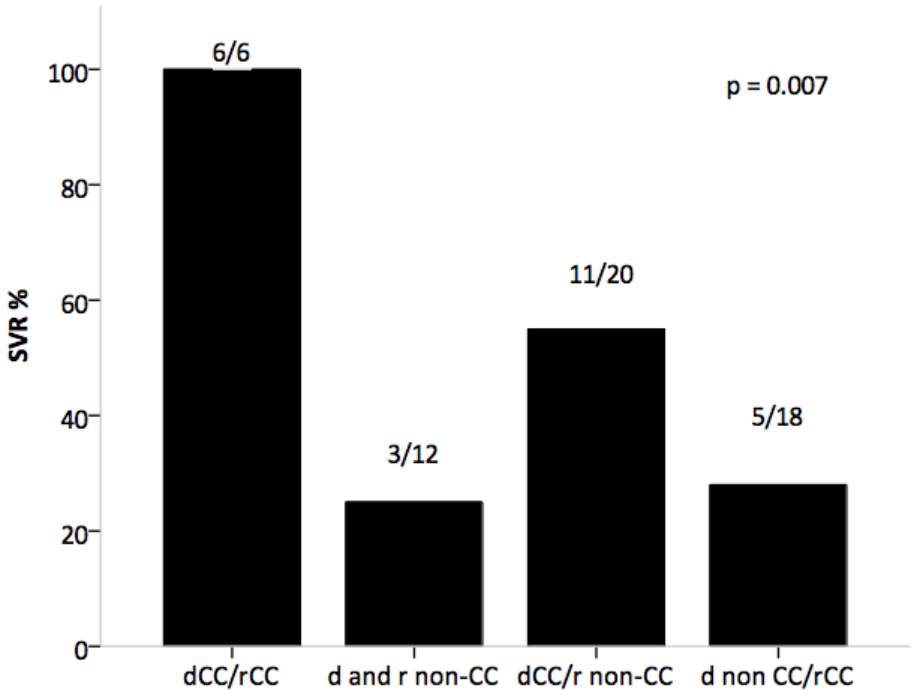


Figure 4.1 Treatment outcome according to rs12979860 IL-28B donor/recipient combination (d, donor; r, recipient).

4. Discussion

Various risk factors predicting HCV recurrence post transplantation are well established. Interactions of recipient phenototypic and genotypic factors have long been postulated. The recently discovered SNP (rs12979860) in the IL-28B (IFN - λ3) gene on chromosome 19 has been well validated as a powerful pre-treatment predictor of SVR pre-transplantation. European and North American centres have all recently published their experience of recipient and donor IL-28B genotypes on outcomes post transplantation for HCV (Charlton et al., 2011, Coto-Llerena et al., 2011, Eurich et al., 2011, Firpi et al., 2013). Our patient cohort at King's College Hospital, London are typical of centres in Northern Europe: male, predominately genotype 1 disease and with co-existing diabetes in the majority.

IFN-λ plays an integral role in the modulation of the adaptive immune system and has strong pro-inflammatory properties. IFN-λ also interacts with natural killer cells and increases CXCL10 production by monocytes, endothelial cells and fibroblasts. CXCL10 is produced directly by hepatic stellate cells and has been shown to be predictive of fibrosis progression post-LT (Berres et al., 2011). Attention has therefore turned to the role of IL-28B genotype on HCV recurrence post transplant. At present, differing data exist regarding the impact of IL-28B genotype on HCV recurrence post transplant exists (Charlton et al., 2011, Eurich et al., 2011). In a study of 220 patients, time to recurrence of HCV (histologic and detectable HCV RNA within serum) was delayed in recipients with the rs12979860 CC IL28B genotype compared to those with CT and TT genotypes (5 year recurrence: 78% vs. 87% vs 100% respectively; p=0.02) (Charlton et

al., 2011). On multivariate analysis, the recipient IL28B C allele was an independent predictor of delayed recurrence of HCV at 2 years (HR 0.6, CI 0.4-0.9, p=0.008) and 5 years (HR 0.6, CI 0.5-0.9, p=0.003). Donor IL28B genotype was not associated with time to recurrence of HCV at 2 years or 5 years. In another study, this time performed in Europe, demonstrated that the mean duration to the development of advanced fibrosis (>F3) was associated with recipient IL28B genotype (CC 65 months vs. CT 46 months vs TT 30 months; p=0.02) (Eurich et al., 2012). Another study, failed to identify the presence of IL28B CC genotype in the donor as a protective factor (Cisneros et al., 2012). In stark contrast, the presence of allele IL28B T in the donor was not associated with a higher risk of sever recurrence in the graft but possibly a protective effect (OR 0.46) although this was not statistically significant. Mechanisms for this observation offered by the authors were sparse but included that other studies which have demonstrated the opposite performed donor genotyping using DNA isolated from implanted liver biopsies which could contain recipient –derived DNA thereby confounding the results (Lange et al., 2011, Coto-Llerena et al., 2011). My results demonstrated that recipient non-CC rs12979860 IL-28B genotype were more likely to develop F≥2 at 12 months and progress to F≥4. Donor CC rs12979860 IL-28B genotypes were also less likely to develop F≥4. Donor DNA was extracted prior to implantation into the recipient and therefore there was no possibility of any recipient DNA confounding the results.

Newly published data has identified a novel SNP at position 469415590 (ss469415590 (TT or ΔG)) upstream of IFN-λ3 gene which may play an integral role in treatment

response (Prokunina-Olsson et al., 2013). Using primary human hepatocytes, the novel SNP (ss469415590 ΔG) resulted in a frame shift mutation and production of the full-length protein designated IFN- λ4. Using several large independent clinical studies, possession of the ss469415590 ΔG allele was associated with a reduced association with spontaneous HCV clearance and reduced response rates to IFN-α based treatment. In particular, the study also suggested that ss469415590 was a more sensitive marker than rs12979860 in predicting treatment response and HCV clearance particularly in African Americans (Prokunina-Olsson et al., 2013). Proposed mechanisms of action include refractoriness to IFN signalling. At present, further studies are required to determine the function of IFN- λ4 and its role in HCV infection and treatment.

Given the slower fibrosis rates associated with recipient and donor IL-28 CC genotypes, I assessed this combination with regards to F≥2 at 12 months and F≥4 post transplantation. My data demonstrated that patients with donor/recipient IL-28B CC combination were less likely to develop F≥2 at 12 months and F≥4 post transplantation. Recipient IL-28B CC vs. non CC genotype was the only significant factor identified that was associated with a rapid fibrosis progression i.e. F≥2 at 12months. Factors associated with the development of F≥4 post transplantation included donor age, diabetes mellitus and recipient IL28B non CC genotype. The use of anti-viral therapy resulting in a SVR and recipient IL-28B CC vs. non CC genotype were both associated with a reduced risk of developing F≥4 post transplantation. The IL28B genotype also appears to correlate with the inflammatory score and fibrosis stage on

liver biopsy (Firpi et al., 2013). The favourable CC genotype being associated with lower HAI scores and less fibrosis compared to the non-CC genotypes. This finding suggests the IL28B SNP affects HCV outcome through interacting with the host immune system.

In keeping with published data, my results demonstrated no association between IL-28B genotype (recipient or donor) and graft or patient survival when censored at 5 years. Studies to date including our own have had limited follow-up and small numbers and are therefore probably not powered sufficiently to address these issues. Studies with longer follow up are therefore warranted.

The IL-28B SNP is an established predictor of response to anti-viral therapy both in the pre- and post-liver transplant period. Certainly the combination of IL-28B CC donor/recipient genotype appears to be associated with a SVR with PR based-therapy. Although only 6 of our patient group carried this combination, all 6 patients achieved a SVR. The data are limited with regards to the number of patients included and therefore require further validation using larger prospective cohorts. However, published data would certainly support a higher SVR rate in individuals with the IL-28B CC donor/recipient genotype (Charlton et al., 2011). Proposed mechanisms for this beneficial response are associated with changes in the immune response and also the level of interferon sensitive gene activation (Honda et al., 2010). The advent of newer oral DAAs, in particular interferon-free regimens, which do not rely on host interferon

sensitivity, will potentially negate the use of IL-28B genotype with regards to predicting treatment response.

The number of patients that underwent AVT were relatively small. These patients had been treated predominately in the later part of the study cohort (after 2005). Consequently, donor and recipient DNA were more readily available and I was also able to extract DNA from stored blood/tissue. To determine whether donor/recipient IL28B genotype was predictive of treatment response, I needed to ensure the numbers included in the analysis were as robust as possible. This may have introduced a potential confounder but my results are in keeping with other published data.

Some authors have suggested that allocation of donor grafts should be to those with the favourable IL-28B CC genotype. My data would certainly suggest a slower rate of fibrosis progression and improved response rates to AVT and SVR rates. In the rapidly changing landscape of DAAs how long this holds true remains to be seen. Clearly this selection policy would also add further strain onto an already pressured donor pool. It is likely that given the evolution of DAA therapy, patients awaiting liver transplantation will undoubtedly be more likely to be treated on the waiting list (McCaughan, 2012). This will hopefully result in more patients entering liver transplantation with an undetectable HCV viral load and consequently reducing the risk of HCV recurrence.

Strengths of this work include stored donor DNA taken at the time of liver transplantation. In addition, recipient DNA was also readily available. The experiments were performed by myself and then validated by Dr James Underhill ensuring that the experiments and interpretation were correct. The results of the donor and recipient IL28B genotype were then interpreted using the HCV post liver transplant database. Weaknesses include DNA degradation and suitable concentrations of both donor and recipient DNA available. All samples were assessed for suitability but unfortunately in some cases further DNA extraction was required. In total, I had to extract recipient DNA for 10 patients from stored samples in order to try and improve the concentration of DNA. Other weakness clearly include the retrospective design which also limited the number of samples available, especially of donor DNA. Another area of interest which was not possible was the determination of donor and recipient IL28B genotype in patients that develop FCH. One would hypothesise that patients that develop FCH would have the donor/recipient rs12979860 TT genotype. At the time of my experiments and analysis, treatment for FCH was very limited and often FCH was a terminal occurrence. FCH now however can be treated and is rare. Patients who remain viraemic entering liver transplantation could be highlighted as high risk for FCH and for early exposure to treatment if the donor and recipient rs12979860 genotype was TT.

In conclusion, my data demonstrates that the recipient rs12979860 IL-28B genotype is a strong predictor of rapid HCV recurrence and fibrosis at 12 months post transplantation. In addition patients that carry the unfavourable CT or TT genotype are

less likely to respond to AVT and are more likely to develop F>4 post LT. The combination of both recipient and donor rs12979860 IL-28B CC genotype is associated with better SVR rates. It is likely that treatment options for this population will evolve rapidly negating the use of interferon based therapies.

Chapter V

The role of CXCL10 in predicting HCV recurrence and treatment response post liver transplantation

1. Background

The role of CXCL10 has been discussed previously in Chapter II. Two of CXCL10 roles appealed to me and led me to conduct the next series of experiments. Firstly, CXCL10 is produced by hepatocytes and HSC, suggesting it may have a role in predicting fibrosis progression. Secondly, CXCL10 is a valid marker of ISG activation. To summarise, lower levels of CXCL10 are associated with a lower level of activation of the innate, interferon associated immune response. Therefore, when exogenous interferons are introduced, CXCL10 and ISGs are up-regulated resulting in higher SVR rates. Having determined, the recipient and donor IL-28B genotype in Chapter IV, it seemed logical to therefore determine the role of CXCL10 alone and in combination with IL-28B genotype. Although there was some data on the use of CXCL10 in predicting treatment response in cohorts before transplantation, there was no data post transplantation at the time of conducting my experiments in 2009.

2. Subjects

Between January 2000 and January 2011, 303 adult patients (> 18 years) with chronic HCV underwent primary liver transplantation at King's College Hospital NHS Foundation Trust, London, UK. Patients were identified using the Institute of Liver Studies viral hepatitis computerized database. Patients with a fibrosing, cholestatic hepatitis (FCH) (n=5), those undergoing re-transplantation (n=13) were excluded along with individuals co-infected with human immunodeficiency virus (HIV, n = 8), chronic hepatitis B virus (HBV, n = 12) and those undergoing joint liver-kidney transplantation

(n=1). Patients that did not survive a minimum of 12 months were also excluded (n=74). 57 patients did not have a liver biopsy performed at 12 months as they were not followed up at our institution. Of the remaining patients, 133 had a 12 month liver biopsy performed. Median number of biopsies per patient was 2 (range 1-4). Patients were considered for AVT once there was evidence of progressive fibrosis (F≥2, Ishak) on liver biopsy. Institutional ethical approval was obtained prior to commencement of the study.

3. Results

3.1 Patient characteristics

133 patients, 111 (83%) male, who had undergone primary liver transplantation for HCV related cirrhosis had a liver biopsy performed 12 months post transplantation and were included in the analysis. HCV genotype 1 (49%) was the most prevalent viral genotype. Median recipient age at time of liver transplantation was 53 years (19-67) and median donor age was 46 years (15-87). The majority (81%) were Caucasian. HCC at the time of transplantation was evident in 50 (38%) patients and a significant alcohol history was evident in 30 (23%) patients. HBV core antibody positivity was evident in 30 (23%) patients. Median follow up was 46 months (12-142).

3.2 Slow (F<2) versus fast (F≥2) fibrosis recurrence at 12 months

Patients were stratified according to their fibrosis stage at 12 months to either a slow (F<2) fibrosis group (n=77) or a fast (F≥2) fibrosis group (n=56). The characteristics of these patients are shown in Table 5.1. Significant differences between the two groups

were male sex, donor age, donor risk-index (DRI), diabetes mellitus (pre-transplant and new onset post-transplant) and necro-inflammatory scores.

	Slow (F<2) N= 77	Fast (F≥2) N= 56	P
Recipient Age (years)*	54, (35-67)	52, (19-67)	0.8
Male (n/%)**	69/90	42/75	0.03
Caucasian (n/%)**	61/79	47/84	0.5
BMI (kg/m^2)*	27, 20-35	25, 20-35	0.167
HBcAb (n/%)**	19/25	11/20	0.51
ALD (n/%)**	16/21	14/25	0.41
HCV genotype 1 (n/%)**	36/48	29/62	0.5
HCC (n/%)**	24/31	26/46	0.07
Diabetes (n/%)**	31/40	38/69	0.001
Donor age (years)*$^+$	41, (15-67)	56, (23-85)	<0.0001
DRI*	1.65, (1.1-2.5)	1.8,(1.2-3.0)	0.006
DBD (n/%)**	59/76	33/60	0.3
CIT (hours)*	9.9 (4.5-18.3)	9.7(5.7-15.5)	0.72
Tacrolimus (n/%)**	74/96	56/100	0.4
ACR (n/%)**	23/30	17/30	0.9
Fibrosis* (Ishak)	1, (0-1)	2, (2-5)	<0.0001
NI*	4, (0-7)	5, (0-10)	<0.0001
CXCL10 (pg/ml)*	112, (51-189)	245, (90-556)	<0.0001
MELD*^	14, (6-40)	13, ((6-24)	0.14
CPS*^	9, (5-14)	9, (5-13)	0.56
Patient Survival (m)*	50, (13-131)	49 (18-143)	0.8
Patients that developed F≥4 ** (n/%)	16/21	32/57	0.001
Time to F≥4*	43, (14-124)	29, (14-120)	0.04

Table 5.1 .Patient characteristics dependent on their fibrosis stage at 12 months (Slow, F<2 versus Fast, F≥2).

ACR, acute cellular rejection; ALD, alcohol related liver disease; BMI, body mass index; CIT, Cold ischaemia time; CPS, Child Pugh Score; DRI, donor-risk index; F, fibrosis; HCC, hepatocellular carcinoma; NI, necro-inflammatory score.

Values expressed as either median with range (min-max) or frequency and %. * Mann Whitney; ** Chi square .

^ at the time of liver transplantation; $^+$ number of donors included was 133

CXCL10 levels were significantly lower in slow fibrosis group (112, 51-189 pg/ml) compared to the fast fibrosis group (245, 90-556 pg/ml, p<0.0001) and differed according to the necro-inflammatory score (Figure 5.1). CXCL10 levels also correlated with fibrosis stage (r=0.86, p<0.0001), necro-inflammatory score (r = 0.52, p<0.0001) serum AST at 6 months (r=0.42, P<0.0001) and serum ALT at 6 months (r=0.67, p<0.0001) (Figure 5.2).

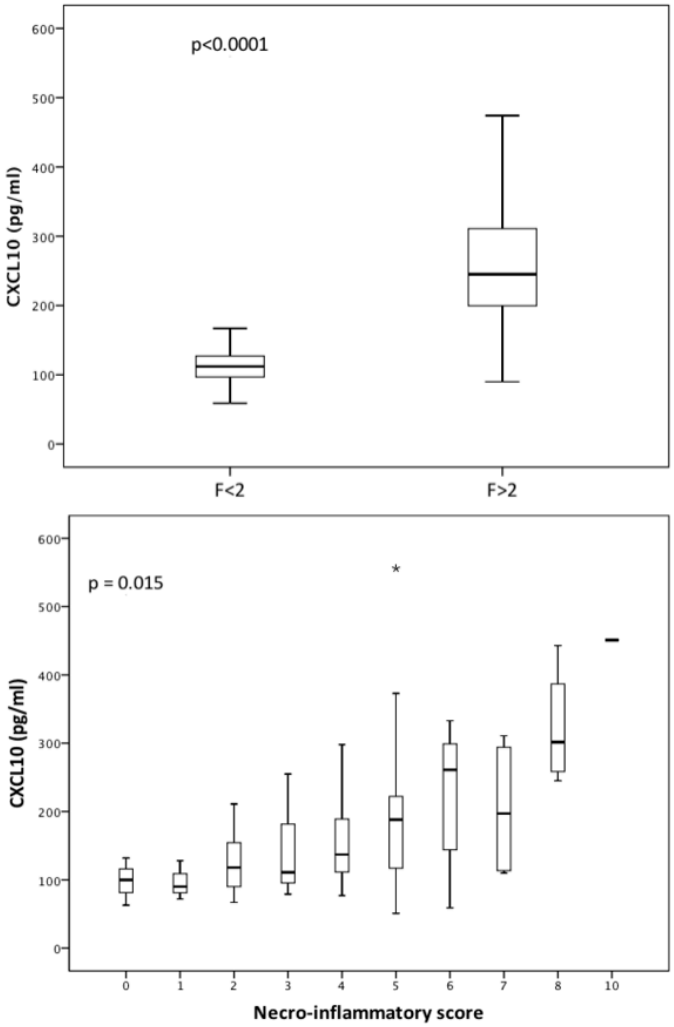


Figure 5.1. CXCL10 levels according to fibrosis and necro-inflammatory score at 12 month liver biopsy: CXCL10 levels according to fibrosis (F) stage (F<2 versus F>2) and necro-inflammatory score.

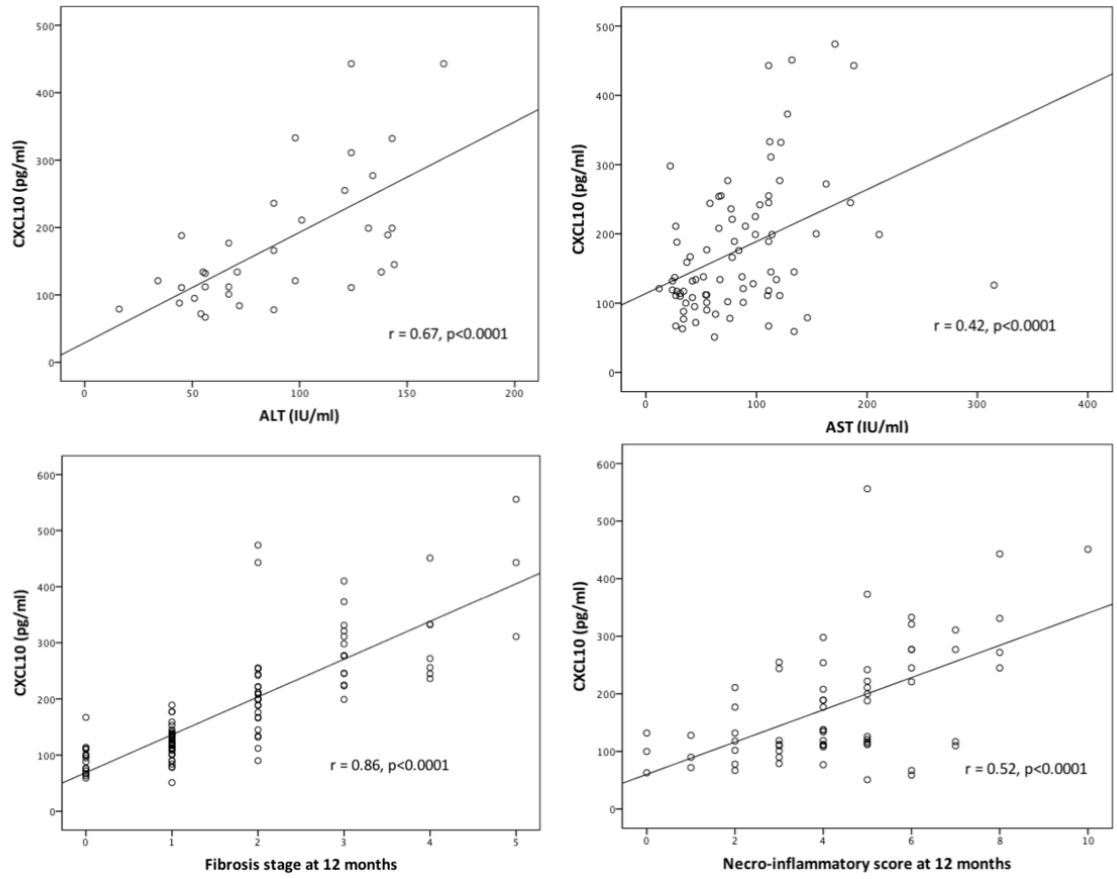


Figure 5.2. Correlation of CXCL10 with fibrosis, necro-inflammatory score, aspartate transaminase and alanine transaminase.

3.3 Predictors of F≥2 at 12 months

CXCLI0 predicted the development of F≥2 at 12 months with high accuracy (Area under curve, AUC 0.95, 95% confidence interval (CI) 0.90 – 0.98, p<0.0001) along with donor age (AUC 0.73, 0.62 -0.85, p = 0.001) and diabetes mellitus (AUC 0.66, 0.53-0.79, p = 0.02) (Figure 5.3). A CXCL10 cut-off value of 163 pg/ml was associated with the highest sensitivity and specificity.

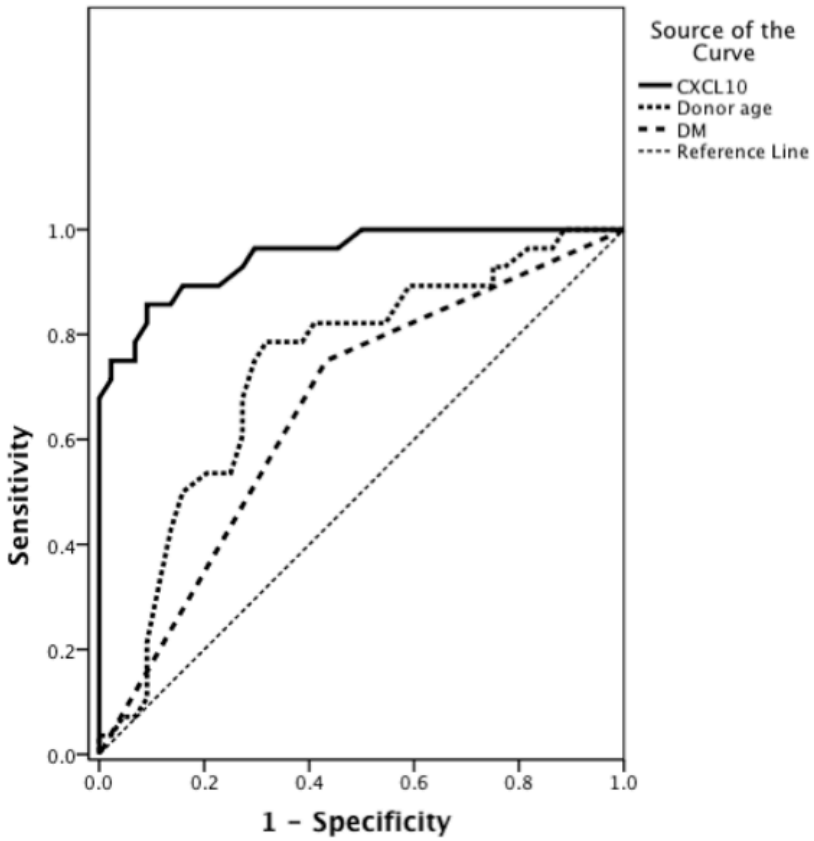


Figure 5.3. Receiver operating curves for performance of CXCL10, donor age and diabetes mellitus (DM) to predict fibrosis stage ≥ 2 at 12 months

In a univariate logistic regression model (Table 5.2), CXCL10, IL28B CC genotype, diabetes mellitus pre-transplantation and donor age were significant predictors of F≥2 at 12 months. In a multivariate logistic regression model, CXCL10 (Odds ratio, OR 1.05, 95% CI 1.03 – 1.08, p = 0.01), IL28B CC genotype (OR 0.3, 0.13-0.66, p=0.03) and donor age (OR 1.07, 1.02-1.14, p=0.05) remained significant. HCV viral genotype was not included as there is little data to suggest it influences fibrosis recurrence at 12 months.

Parameter	Odds ratio	95% Confidence Interval	P value
Univariate			
Donor age	1.07	1.03-1.12	<0.0001
Diabetes mellitus	3.3	1.64-7.24	0.001
IL28B CC genotype	0.6	0.13-0.67	0.003
CXCL10	1.5	1.3 -1.77	0.0001
Multivariate			
Donor age	1.07	1.02-1.14	0.05
Diabetes mellitus	2.43	0.4-16.7	0.3
IL28B CC genotype	0.3	0.13-0.66	0.03
CXCL10	1.05	1.03-1.08	0.01

Table 5.2. Factors predictive of F≥2 at 12 months.

3.4 Risk factors for F≥4 post transplant

Sixteen patients (21%) who had F<2 at 12 months went onto develop F≥4 post transplantation compared to 32 patients (57%) who had F≥2 (p=0.001) (Figure 5.4). Diabetes mellitus was the strongest predictor of F≥4 post transplant (AUC 0.86, 0.76-0.95, p<0.0001 compared to CXCL10 (AUC 0.79, 0.68-0.89, p<0.0001) and donor age (AUC 0.65, 0.52-0.78, p =0.04) (Figure 5.5).

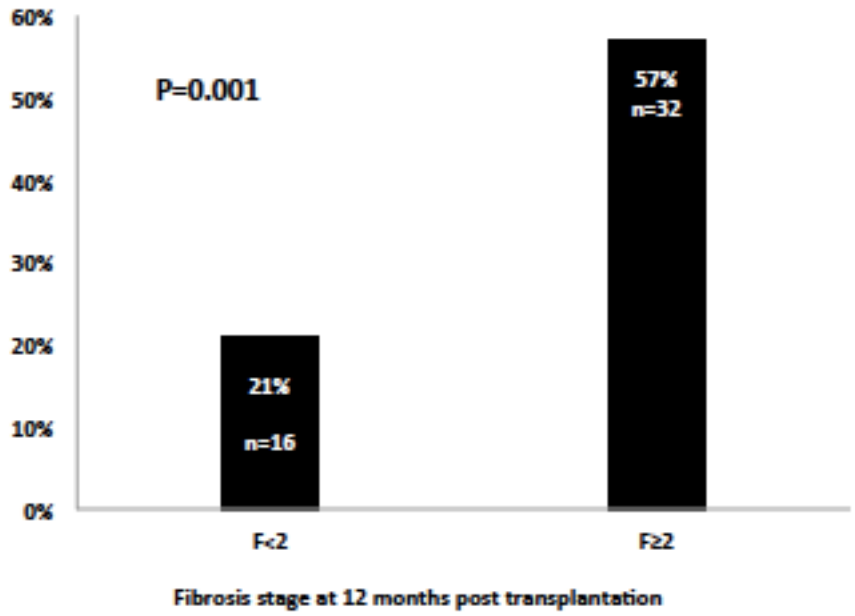


Figure 5.4. The development of F≥4 post liver transplantation according to F<2 or F F≥2 at 12 months.

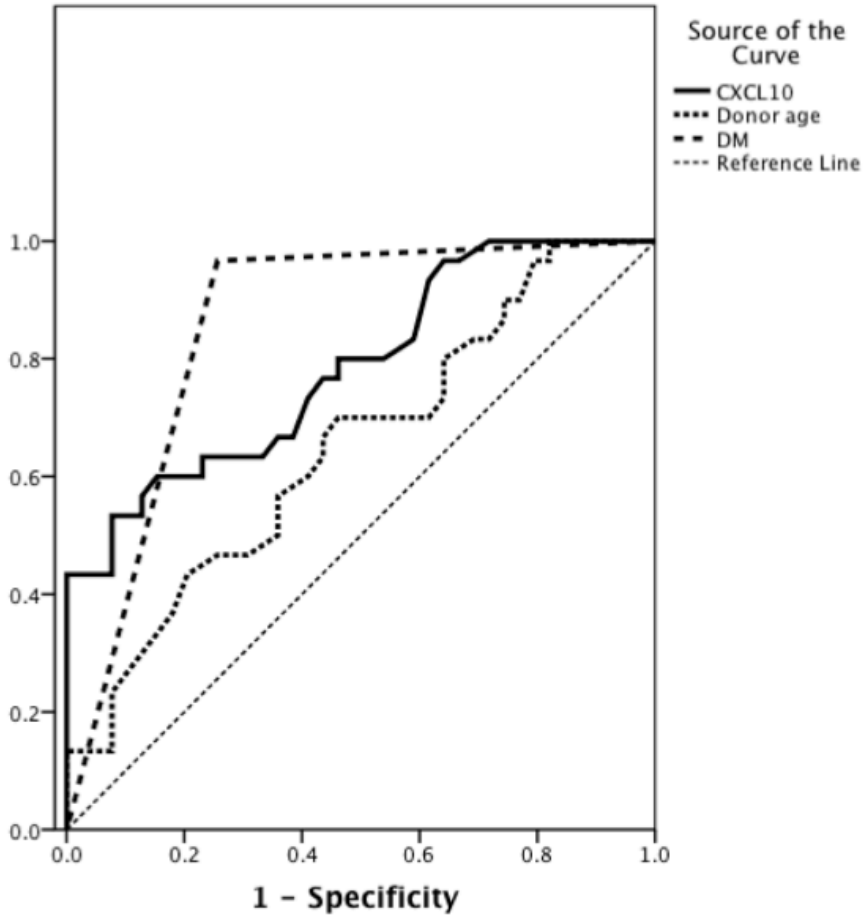


Figure 5.5. Receiver operating curves for performance of CXCL10, donor age and diabetes mellitus (DM) to predict F ≥ 4 post liver transplantation.

Using a univariate Cox regression model, CXCL10, diabetes mellitus post transplantation, donor age and HCV genotype 1 influenced the time to F≥4 post transplantation (Table 5.3). On multivariate analysis, diabetes mellitus remained a strong predictor of F≥4 (HR 2.4, 1.01-5.76, p=0.04) along with CXCL10 (HR 2.5, 1.03-5.3, p=0.04) and donor age (HR 2.2, 1.06-7.9, 0.05). Patients with CXCL10 level > 163

pg/ml had a quicker time to development of F≥4 post transplantation (Log rank p < 0.0001, Figure 5.5).

Parameter	Odds ratio	95% Confidence interval	P value
Univariate			
Donor age	2.5	1.4-4.4	0.002
Diabetes mellitus	2.6	1.4-5.0	0.003
Recipient age	0.9	0.9-1.01	0.14
HCV genotype 1	2.1	1.02-4.2	0.05
CXCL10	3.3	2.0-7.7	<0.0001
Multivariate			
Donor age	2.2	1..06-7.9	0.05
Diabetes mellitus	2.4	1.04-5.8	0.04
Recipient age	-	-	-
HCV genotype 1	1.6	0.65-4.1	0.31
CXCL10	2.5	1.03-5.3	0.04

Table 5.3 Factors predictive of F≥4 post liver transplantation

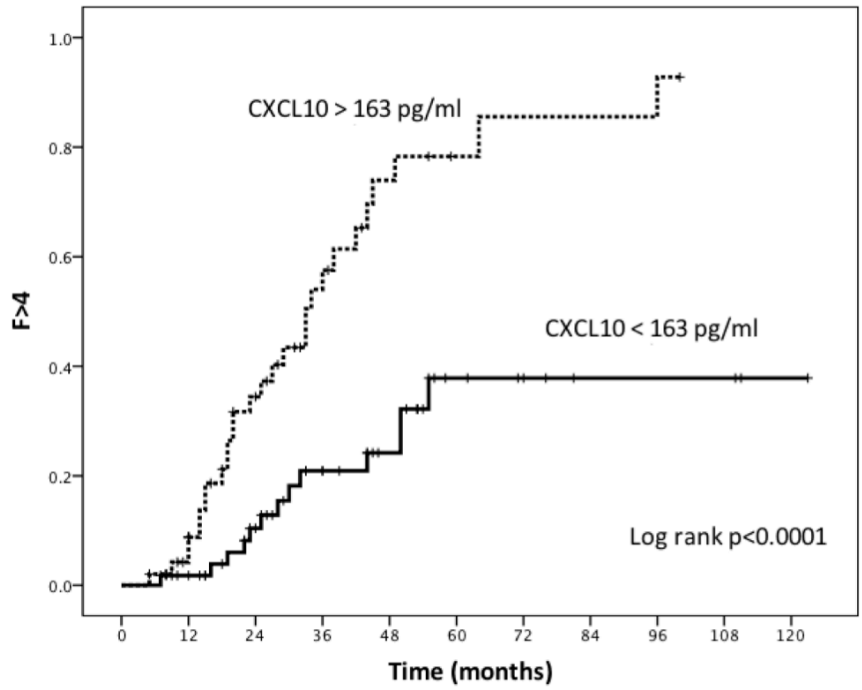


Figure 5.6 Cumulative risk for development of F≥4 post transplantation according to CXCL10 levels measured at 6 months post transplant

3.5 Patient and graft survival

Using the CXCL10 cut-off of <163 pg/ml, no differences in patient survival at 12 (98% versus 98%), 36 (89% versus 87%) and 60 (79% versus 71%) months were observed respectively (Log rank p = 0.5). There was a trend towards improved graft survival in patients with a CXCL10 level < 163pg/ml at 12 (98% versus 98%), 36 (90% versus 80%) and 60 (82% versus 63%) months respectively but this was not statistically significant (Log rank p = 0.08).

3.6 Anti-viral therapy post transplant

Demographic and clinical data of 91 patients that underwent anti-viral therapy for recurrent HCV post-transplantation is shown in Table 5.3. Median time to treatment was 32 months (15-166 months). Median CXCL10 levels taken immediately pre-treatment (time 0) were 272 pg/ml (74-614). 12 patients had been treated with PEG and RBV pre- transplantation unsuccessfully (5 x RR, 6 x NR and 1 intolerant to therapy due to visual disturbance). 71 (78%) patients were commenced on a LADR protocol (median dose of PEG-IFN 135mcg/week, median dose of ribavirin 800mg/day). 80% achieved full dose of PEG-IFN whilst only 73% of patients achieved the full target dose of ribavirin. Median treatment duration was 48 weeks (range 1-72). 12 patients (13%) experienced PEG induced graft dysfunction requiring augmentation of immunosuppression. No patients developed acute cellular rejection and required boluses of corticosteroids or interruption of anti-viral therapy. Baseline CXCL10 levels correlated with serum AST ($r=0.4$, $p=0.001$), ALT ($r=0.4$, $p=0.006$), Fibrosis (F) score on liver biopsy ($r=0.3$, $p=0.02$) and necro-inflammatory (NI) score on liver biopsy ($r=0.7$, $p<0.0001$). CXCL10 levels varied according to recipient IL28B genotype (CC, 132pg/ml vs. CT, 250 pg/ml vs. TT, 532 pg/ml, $p<0.0001$).

	Total cohort (n=91)	Non Genotype 1 (n=43)	Genotype 1 (n=48)	P value*
Recipient age (years)	55(36-67)	54(40-63)	55(36-67)	0.5
Donor age	51(32-71)	52(32-71)	50(36-65)	0.5
Sex (M/F)	55/15	23/7	32/8	-
MELD	8(6-21)	8(6-9)	9(6-21)	0.49
ALT (IU/ml)	109(32-383)	93(32-383)	119(32-325)	0.17
AST (IU/ml)	91(27-388)	81(27-267)	111(28-388)	0.34
Bilirubin (mg/dL)	13(5-127)	12(6-18)	15(5-127)	0.8
HCV viral load (IU/ml)	2.23E6 (1.12E4-3.76E7)	2.23E6 (1.12E4-3.76E7)	2.25E6 (2.1E5-1.35E7)	0.4
Recipient rs12979860 IL-28B genotype (CC/CT/TT)	18/54/15	10/29/0^	8/25/15	-
Time to treatment (months)	32(15-166)	34(15-166)	33(18-156)	0.4
NI score on liver biopsy	6 (2-11)	5 (1-9)	7(2-11)	<0.0001
F score on liver biopsy	3(2-6)	3(2-6)	4(3-6)	0.002
F≥4 (%)	42	27	53	0.008
CXCL10 (pg/ml)	272(74-614)	132(97-612)	400(74-614)	<0.0001

Table 5.4. Baseline demographic, clinical and biochemical data parameters in patients that underwent anti-viral therapy (expressed as median, minimum and maximum)

*Chi-squared; Performed between non-genotype 1 and genotype 1 patients.
^ Recipient rs12979860 IL-28B only available in 39 patients

3.7 Virologic response

Overall, 35 patients achieved an EVR (10 genotype-1 patients and 25 non-genotype-1 patients) (Table 5.5). CXCL10 levels were significantly lower in those that achieved an EVR compared to those that did not (134 vs. 422 pg/ml, p=0.004). 26 patients (29%) achieved SVR (18 non genotype-1 patients and 8 genotype-1 patients. 45 patients (49%) were NR and 20 patients (22%) were RR. Treatment was terminated due to side effects in 23 (25%) patients. The commonest side effects were neutropenia and sepsis. 13 patients required admission for red blood cell transfusion. In the total patient cohort, CXCL10 levels were significantly lower in those that achieved SVR (121 vs. 374 pg/ml, p<0.0001) compared to those that did not. CXCL10 levels were higher in patients who had a NR compared to those who were RR or those who achieved a SVR (441 vs. 202 vs. 121 pg/ml, p<0.0001).

In genotype-1 patients, 32 patients (68%) were NR and 8 (17%) were RR. No significant differences were found in patients that achieved SVR compared to those that did not achieve SVR with regards to recipient age, baseline HCV viral load, MELD score, fibrosis score and percentage that were cirrhotic. AST (55, 28-88 vs. 121, 30-388 IU/ml, p=0.014), ALT (55, 32-100 vs. 124, 35-325 IU/ml p=0.005), NI scores on biopsy (3,2-8 vs. 8, 3-11, p=0.003) and CXCL10 levels were significantly lower in patients that achieved SVR (106, 74-134 vs. 411,178-614 pg/ml, p<0.0001). CXCL10 levels also varied according to treatment response (NR, 441 vs. RR, 231 vs. SVR, 106 pg/ml, p<0.0001).

In non genotype-1 patients, 10 patients (23%) were NR and 15 patients (35%) were RR. CXCL10 levels were lower in patients that achieved SVR (122, 99-144 vs 220, 97-612 pg/ml, p=0.004). CXCL10 levels were again highest in the NR group compared to RR and those that achieved SVR (385 vs. 201 vs. 122, p=0.002).

	Total Cohort (n=91)	Non Genotype 1 (n=43)	Genotype 1 (n=48)
EVR (n/%)	35/39	25/58	10/21
SVR (n/%)	26/29	18/42	8/17
CXCL10 (pg/ml) according to SVR (Yes vs. No)	121 vs. 374**	122 vs. 220*	106 vs 411**
NR (n/%)	45/49	10/23	32/68
RR (n/%)	20/22	15/35	8/17
CXCL10 (pg/ml) according to treatment response (NR vs. RR vs. SVR)	441 vs.202 vs. 121**	385 vs. 201 vs. 122*	441 vs. 231. 106**

Table 5.5. Treatment response and CXCL10 levels.
EVR, early virologic response; SVR, sustained virologic response; NR, non response; RR, responder relapser
* p<0.05, ** p<0.0001

3.8 Predictors of sustained virologic response

Univariate logistic regression analysis identified non-genotype-1, EVR, adherence to treatment, fibrosis score <4, IL28B CC genotype and pre-treatment CXCL10 levels to be predictors of a SVR (Table 5.6). Only HCV non genotype-1, fibrosis < 4, EVR, IL28B CC genotype and CXCL10 levels remained significant predictors of a SVR on multivariate analysis (Table 5.6). For the total cohort, ROC analysis identified a cut-off of 126 pg/ml

(AUROC 0.96, 95% CI 0.9-1.0, p<0.0001) with a sensitivity of 94% and specificity of 55% for predicting a SVR.

Parameter	Hazard ratio	95% CI	P value
Univariate			
Recipient age	0.9	0.9-1.02	0.2
Non Genotype-1	5.4	1.8-16.9	*0.003*
EVR	1.7	1.28-2.38	*<0.0001*
Adherence to treatment*	10.7	1.32-86.6	*0.03*
HCV VL <800,000	0.3	0.087-1.32	*0.12*
Fibrosis <4	4.9	1.273-19.0	*0.02*
Recipient rs12979860 IL-28B CC genotype	1.6	4.2-7.7	*0.003*
CXCL10	1.2	1.02-1.9	*0.003*
Multivariate			
Non Genotype-1	4.1	3.1-22.6	*0.007*
EVR	2.5	1.5-10.2	*0.009*
Fibrosis < 4	3.1	2.3-26.1	*0.04*
CXCL10	1.3	1.3-2.1	*0.04*
Recipient rs12979860 IL-28B CC genotype	2.0	1.1-5.0	*0.04*

Table 5.6. Predictors of sustained virologic response

* Adherence to treatment determined as > 80% treatment duration

3.8.1 Genotype-1 patients

CXCL10 levels differed according to recipient rs129870 IL28B genotype (CC, 132 pg/ml vs. CT, 250pg/ml vs. TT, 532 pg/ml, p<0.0001). In genotype -1 patients (n=48) alone, ROC analysis identified a cut-off of 154 pg/ml (AUROC 0.84, 95% CI 0.71-0.97, p<0.0001) with a sensitivity of 95% and a specificity of 79% for predicting a SVR. To determine the performance of the recipient rs12979860 IL28B genotype alone and then in conjunction with pre-treatment CXCL10 levels <154 pg/ml for genotype-1 patients respectively, the sensitivity, specificity, positive predictive value (PPV) and the negative predictive values (NPV) were calculated. In genotype-1 patients, using non TT (CC or CT) vs. TT recipient rs12979860 IL28B genotype to predict SVR, the sensitivity, specificity, PPV and NPV were 23%, 100%, 100% and 41% respectively. The addition of an CXCL10 level < 154 pg/ml significantly increased the sensitivity and NPV from 23% to 55% and 41% to 86% respectively (<0.0001).

	Sensitivity %	Specificity %	PPV %	NPV %
Non TT vs. TT recipient rs12979860 IL28B genotype	23	100	100	41
Non TT vs. TT recipient rs12979860 IL28B genotype and CXCL10 level < 154 pg/ml	55*	100	100	86*

Table 5.7. Performance of rs12979860 IL28B genotype and CXCL10 in predicting a sustained virologic response in genotype 1 patients

PPV, positive predictive value, NPV, negative predictive value

* Comparative increase in sensitivity and NPV, p<0.0001

4. Discussion

Identifying patients early with rapid fibrosis progression or more importantly who are deemed at risk for developing rapid fibrosis seems a sensible management strategy. These patients would therefore be candidates for early AVT also. A liver biopsy performed at 12 months readily identifies patients with a fast fibrosis (F≥2) phenotype. A fibrosis stage ≥2 at 12 months has also been identified as an important factor associated with inferior patient survival post-transplantation (Gallegos-Orozco et al., 2009). In our study, we evaluated the role of CXCL10 as a predictor of the development of slow fibrosis (F<2) and fast fibrosis (F≥2) progression at 12 months, and the development of F≥4 post transplantation in patients with HCV recurrence. Our results, in keeping with previous studies, demonstrated CXCL10 levels taken at 6 months post transplant were predictive F≥2 at 12 months with a high degree of accuracy and also predicted the development of F≥4 post-transplantation. An CXCL10 level > 163 pg/ml at 6 months post LT was identified as a significant predictor for the development F≥4 and was associated with a quicker time to the development of F≥4 post-transplant. Figure 5.5 demonstrates the cumulative risk for the development of F≥4 according to CXCL10 at 6 months.

There was certainly a trend towards improved survival in patients with CXCL10 < 163 pg/ml which may become evident with longer follow-up. This finding is important as CXCL10 levels taken at 6 months post transplant could therefore identify patients with

a more aggressive fibrosis phenotype and individuals who would require AVT earlier. This finding needs to be validated using larger cohorts.

Given that CXCL10 is produced by endothelial cells, monocytes and by HSCs attention has therefore turned to assessing the role of CXCL10 in predicting fibrosis progression. Our results are in keeping and confirm a recent study that evaluated the role of CXCL-10 amongst other chemokines in predicting fibrosis progression in HCV patients-post transplant (Berres et al., 2011). In a study of 90 patients with predominately genotype 1 disease, the authors demonstrated significant correlation of CXCL10 levels with the severity of fibrosis and necro-inflammatory activity at 1-year post transplantation and found CXCL10 to be a significant predictor of fibrosis stage at year 3 post-transplantation (Berres et al., 2011). An CXCL10 value of > 140 pg/ml was predictive of the development of F2 fibrosis ($p = 0.0013$) whilst a level of > 220 pg/ml predicted the development of F3 fibrosis ($p = 0.04$). Given the small numbers of patients (n = 10) that developed F4 during the follow-up period, life table analysis was targeted towards those that developed $F \geq 3$. Our study however differs as we aimed to establish whether CXCL10 levels at 6 months post transplant were predictive of $F \geq 2$ at 12 months and the development of $F \geq 4$.

CXCL10 is a pro-inflammatory chemokine responsible for the recruitment of activated lymphocytes to the liver. It would therefore appear counter-intuitive why elevated levels of CXCL10 would be associated with a poor treatment response. One hypothesis suggested is that a predominately antagoistic form of CXCL10 is present (Casrouge et

al., 2011). Dipeptidyl peptidase IV (DPP4 or CD26), expressed by hepatocytes, fibroblasts and endothelial cells, truncates CXCL10. The resultant negative form of the protein can bind CXCR3 but does not induce signalling (Casrouge et al., 2011). Studies have been performed in patients with acute HCV which have measured the truncated form of CXCL10, DDP4 activity and the functionality of HCV- specific T cells and NK cells. (Riva et al., 2014). The data demonstrated that elevated total CXCL10 levels were associated with inferior treatment outcomes resulting in chronic infection. DDP4 activity correlated with concentrations of truncated CXCL10 ($r=0.53$, $p<0.001$) and lower plasma concentrations of truncated CXCL10 and decreasing DDP4 activity were therefore a feature of spontaneously resolving HCV infection (Riva et al., 2014). The same study also demonstrated a higher frequency of HCV-specific IFN-γ producing T-cells and more cytotoxic CD56 dim NK cells and less cytokine-producing CD56 bright NK cells in those who spontaneously resolved infection. These data could be applied to post liver transplant cohorts in a similar fashion to determine whether the truncated form of CXCL10 results in impaired host immunity and HCV infection persistence. Further studies, analysing the expression of CXCR3, the CXCL10 receptor on NK and T cell populations would provide further insights into the pathogenesis of HCV post transplantation.

As eluded to earlier, different forms of CXCL10 exist. Using standard ELISA and Luminex assays, studies have measured total CXCL10 and truncated CXCL10 levels. Single molecule array (Simoa) technology are now available and can potentially overcome this limitation and provides increased sensitivity (Rissin et al., 2010). Total, long and truncated forms of CXCL10 can therefore be measured and studied.

In our cohort, CXCL10 levels varied according to IL28B genotype; higher values noted in CT and TT genotypes. This relationship was statistically significant and has been demonstrated in other cohorts albeit in pre liver transplantation cohorts (Fattovich et al., 2011). The strength of the relationship demonstrated in my data is probably due to the combination of CXCL10 being a marker of fibrosis and ISG upregulation. CXCL10 levels vary according to fibrosis stage as demonstrated earlier, and the median fibrosis stage pre treatment was F3. Median pre-treatment CXCL10 levels were 272 pg/ml at a median time of 32 months post transplant. A pre-treatment CXCL10 level < 126 pg/ml was associated with a SVR. In genotype 1 patients alone, the cut-off value was 154 pg/ml. Established pre-treatment predictors of SVR with PR include a low-baseline HCV viral load, non-genotype 1 disease, female gender and the absence of advanced fibrosis (F<4) (Watt et al., 2009). In genotype-1 patients, the addition of a recipient non TT rs12979860 IL28B genotype and an CXCL10 level < 154 pg/ml significantly improved the negative predictive value from 41% to 86% ($p<0.0001$). This data could therefore identify patients who more likely to respond and benefit from AVT. At the time of conducting these experiments, DAA therapy was not readily available. Minimising patients to the long duration and potential harmful side-effects of pegylated interferon based therapy were therefore very relevant.

EVR was a significant predictor of success with AVT but data regarding a RVR was limited partly due to the use of a LADR regimen. Treatment currently is with dual therapy (PR) but is associated with inferior SVR rates, an increased side-effect profile including the development of interferon mediated graft dysfunction and is poorly

tolerated compared to patients pre-transplantation (Watt et al., 2009). The use of the newer directly acting anti-viral agents (DAA) in post-transplant patients is limited but preliminary data is encouraging although meticulous attention is required to avoid drug-drug interactions between calcineurin inhibitors and the protease inhibitors (Coilly et al., 2012). For the immediate future, AVT appears to be with a PEG/RBV backbone suggesting a possible role for CXCL10.

Our study has several limitations related to its retrospective and mono-centric design. Another potential source of bias is that although we excluded certain patient groups (HBV, HIV co-infected, patients undergoing re-transplantation, those that underwent a combined liver-kidney procedure and those that did not survive 12 months), a further 57 patients predominately transplanted in the early part of the study were not eligible for inclusion as they had not undergone a liver biopsy at 12 months. It is likely that these patients will have had unrecognised fibrosis and may have added to the number of patients with fast fibrosis progression at 12 months and F≥4 post-transplantation. Measurement of CXCL10 in patients with FCH would have provided interesting data on the host immune response. Our numbers were small and our results require further validation in larger cohorts especially using the CXCL10 cut-off values we describe.

In conclusion, CXCL10 levels measured at 6 months post transplant predict the development of rapid fibrosis at 12 months (F≥2) and F≥4 post-transplantation in patients with recurrent HCV. In addition, pre-treatment CXCL10 levels identify patients that are likely to respond to AVT. These results may therefore help identify patients

with rapid fibrosis and those requiring early exposure to AVT in the post-transplant period but also importantly, those that are likely to respond successfully.

Chapter VI

Defining the role of microRNA in predicting HCV recurrence post liver transplantation and their role as biomarkers for HCV recurrence.

1. Background

miRNAs have been shown to play an essential role in HSC function, activation and hepatic fibrosis (Moreira, 2007, Atzori et al., 2009). To date, there are a paucity of studies performed in the post-transplant setting especially in patients with recurrent HCV. HCV recurrence post liver transplantation represents a unique model of accelerated fibrosis and therefore I aimed to delineate possible pathways involved. In addition, I aimed to identify possible miRNAs could act as serum biomarkers for fibrosis. Finally, I aimed to delineate the different miRNA profiles involved in acute cellular rejection (ACR) and recurrent HCV.

2. Subjects

All adult patients (> 18 years) transplanted for HCV related cirrhosis that received a heart beating donor between January 2000 and January 2010 at King's College Hospital, London were identified. Protocol liver biopsies are performed at our Institution at 12 months or earlier in the event of graft dysfunction. Specimens were assessed and scored according to the modified Ishak classification (0 - no fibrosis and 6 – cirrhosis). A fibrosis score ≥ 2 at 12 months classified the patient as a fast- progressor with regards to HCV recurrence (Firpi et al., 2004).

Patients with recurrent HCV post-transplant were divided into 2 groups according to their fibrosis score; Group A, slow- progressors (n = 11) – F <2 at 12 months; and Group B, fast- progressors (n=9) - F ≥ 2 at 12 months. Patients in Groups A and B were matched according to the following variables: recipient age, donor age, MELD score at transplant, donor-risk index (DRI), cold – ischaemia time (CIT), presence of diabetes,

evidence of CMV viraemia, and immunosuppression regimen. Patients were selected prior to the miRNA expression studies and were identified from a total cohort of 133 patients. All patients received dual therapy with tacrolimus (target trough levels 8-10 mg/ml) and a reducing dose of corticosteroid following transplantation. No patient received OKT-3 or an interleukin-2 blocker. Data capture included post-operative clinical course, immunosuppression, number of methylprednisolone boluses, occurrence of biopsy-proven acute cellular rejection and serum liver graft function tests. Group C (n = 5) consisted of biopsies taken from patients with HCV and acute cellular rejection (according to the Banff criteria). Group D (n = 4) consisted of biopsies taken from patients transplanted for alcohol related liver disease and served as the control group. All biopsies were performed and driven by clinical necessity. Exclusion criteria included hepatocellular carcinoma at time of transplant, co-infection with hepatitis B virus or human immunodeficiency virus types 1 and 2, and liver disease of other origin than HCV infection (except for patients in Group D). Stored serum from patients in group A, slow – progressors and group B, fast progressors were analysed for miRNA identified using quantitative PCR analysis. Serum samples were taken at median of 6 months post transplantation (range 4-10 months)

3. Results

In total, 29 patients were included in the analysis: patient demographics and clinical data are listed in Table 6.1. As expected genotype 1 disease was the most prevalent and represents the HCV population seen in our centre. The recipient rs12979860 IL-28B TT haplotype was equally represented across Groups A, B and C. Median HCV viral loads were also comparable. Representative examples of the histology are shown in Figure 6.1.

TABLE 1. Patient Demographics for Slow Fibrosis Progressors (Group A), Fast Fibrosis Progressors (Group B), HCV and ACR Patients (Group C), and Controls (Group D)

	Patient Groups*			
	A (n = 11)	B (n = 9)	C (n = 5)	D (n = 4)
Recipient age at transplant (years)†	52 (36-62)	46 (35-57)	45 (26-56)	50 (26-62)
Donor age (years)†	45 (44-58)	46 (29-73)	55 (29-62)	59 (55-69)
MELD score at transplant †	15 (11-19)	14 (11-18)	14 (11-21)	9 (8-14)
HCV genotype 1a (%)	55	56	40	N/A
IL-28B rs12979860 TT genotype (%)	18	22	20	N/A
Pretransplant HCV viral load†	4.77E5 (2.145-9.98E5)	1.84E5 (8.58E4-5.11E5)	2.1E5 (8.558E4-9.98E5)	N/A
Methylprednisone boluses (n)	4	3	5	3
Cold ischemia time (hours)†	9.0 (7.6-15.3)	10.8 (7.8-18.9)	8.8 (7.9-19.2)	14.5 (12.8-15.6)
Biopsy-proven ACR (%)	18	11	100	25
Donor risk index†	1.6 (1.2-2.3)	1.7 (1.5-2.1)	2 (1.8-2.4)	1.8 (1.4-2.1)
Diabetes (%)	45	44	40	50
Cytomegalovirus viremia (%)	18	11	0	25
Variables at biopsy				
Time from transplant (months)†	12 (8-15)	15 (8-17)	8 (5-90)‡	20 (10-56)
Tacrolimus monotherapy (%)	64	77	100	100
Aspartate aminotransferase (IU/mL)†	55 (17-362)	62 (21-263)	227 (105-325)	77 (38-257)
Bilirubin (mg/dL)†	12 (8-23)	17 (7-44)	73 (48-171)	16 (9-32)
Fibrosis score (0-6)†§	1 (0-1)	3 (3-4)	0 (0-1)	N/A
HCV viral load (IU/mL)†	1.25E6 (1.26E4-9.89E6)	5.11E5 (2.6E3-1.21E7)	2.31E5 (4.41E4-3.45E6)	N/A

*There was no statistical difference between groups A and B.
†The data are expressed as medians and ranges.
‡The data are expressed in days.
§Fibrosis score 0-6.

Table 6.1. Patient demographics for Groups A-D.

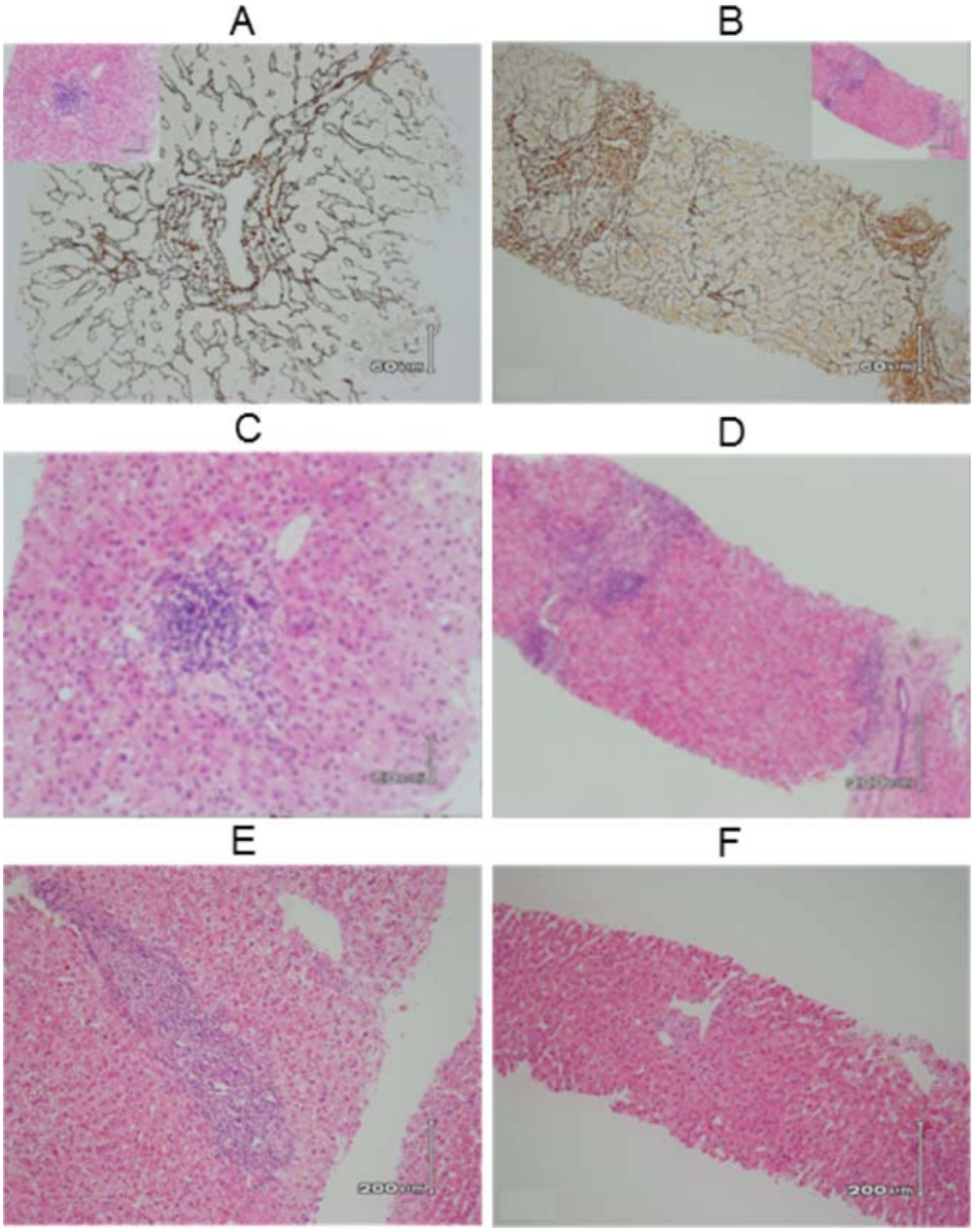


Figure 6.1 Histological analysis of slow fibrosis progressors (group A), fast fibrosis progressors (group B), ACR patients (group C) and controls (group D). (A,B) Representative reiculin staining for (A) group A and (B) group B. (C-F) Representative H&E staining for (C) group A, (D) group B, (E) group C and (F) group D.

3.1 miRNA expression distinguishes slow and fast fibrosis progressors of HCV post-transplant as well as acute cellular rejection

Principal component analysis (PCA) resolves a multidimensional data set by identifying key variables that explain the observed differences. Intragraft miRNA in all 29 patients included in our study was resolved in to three principal components represented in the three-dimensional scatter plot. This demonstrated a clear segregation of all 4 groups according to miRNA expression profiles at a *P* value of 0.01 (Figure 6.2B). The supervised hierarchical cluster analysis (HCA) for miRNA expression demonstrated that a greater similarity of expression existed in each group (Figure 6.2A). The graphical tree (cladogram) above the heat map also confirms this observation, as it reveals stronger clustering within each group than between different groups. It also demonstrates a greater degree of similarity between Groups A and B than the other two groups by miRNA expression. The unequivocal separation of patient groups by both PCA and HCA analysis clearly demonstrates that these groups are functionally distinct in relation to miRNA expression.

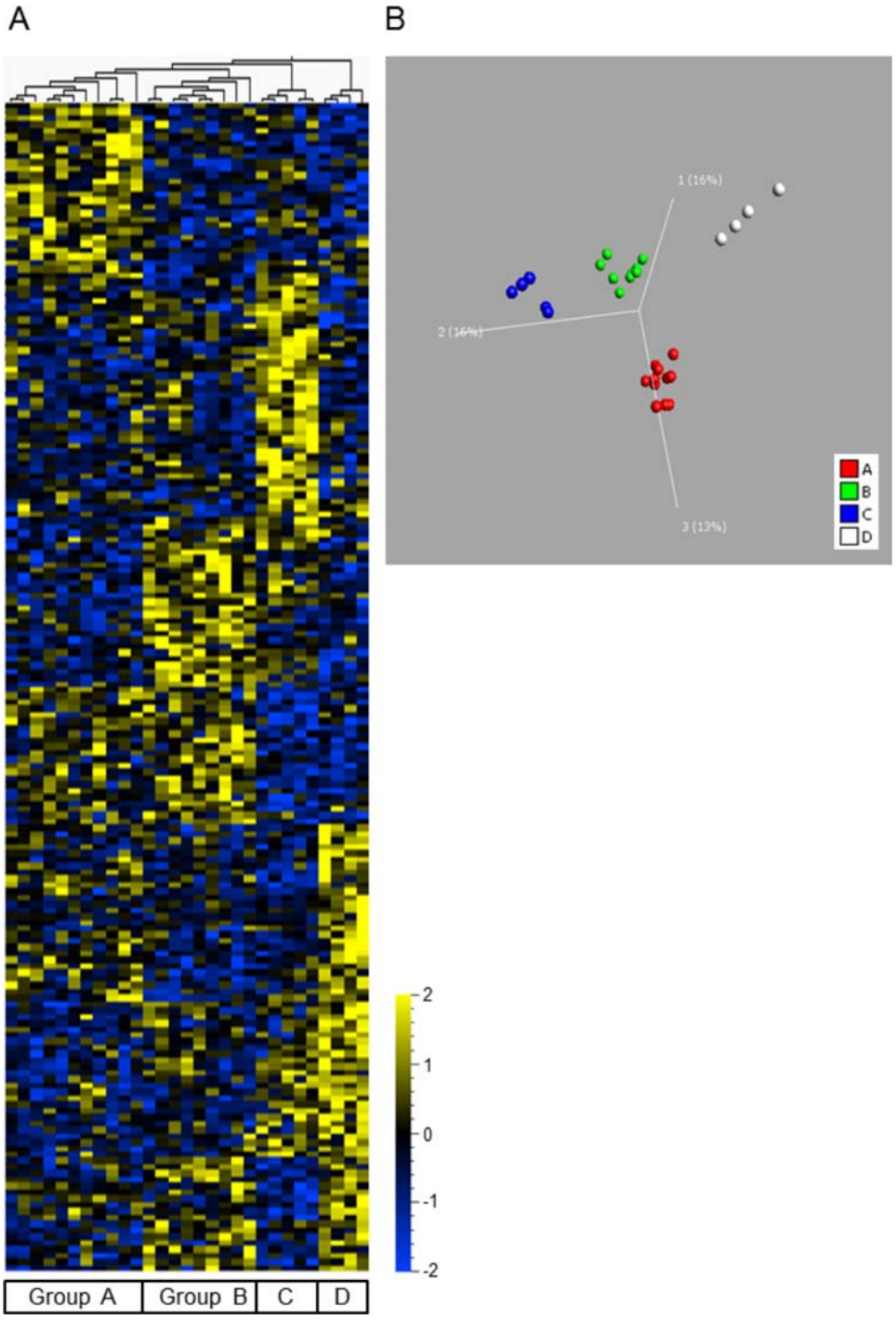


Figure 6.2: **miRNA expression distinguishes Groups A-D. (A) Hierarchical cluster analysis for Groups A-D (*P*<.01). The cladogram shown above the heatmap demonstrates the degree of similarity between the samples in terms of miRNA expression. (B) Principal Component Analysis for the same data-set.**

3.2 miRNA expression distinguishes between slow- and fast- progressors of HCV post-transplant

In order to determine whether there was a difference in miRNA expression profiles between patients with slow and fast fibrosis, we refined our analysis by directly comparing Groups A and B. Patients in both groups were matched for factors that are known to influence the rate of HCV recurrence. Therefore, as expected there was no demonstrable statistical difference between the two groups (see Table 6.1). At the time of liver biopsy, patients were clinically well with satisfactory liver graft function. Median HCV viral loads were comparable both pre- and post-liver transplantation. No patient had been exposed to anti-viral therapy in the post-transplant period at the time of liver biopsy. PCA and HCA performed to a value of 0.01 demonstrated a clear segregation of the 2 groups with respect to miRNA expression (Figure 6.3). 152 miRNAs of statistical significance were identified that were differentially regulated between Groups A and B (Appendix II, Table 1).

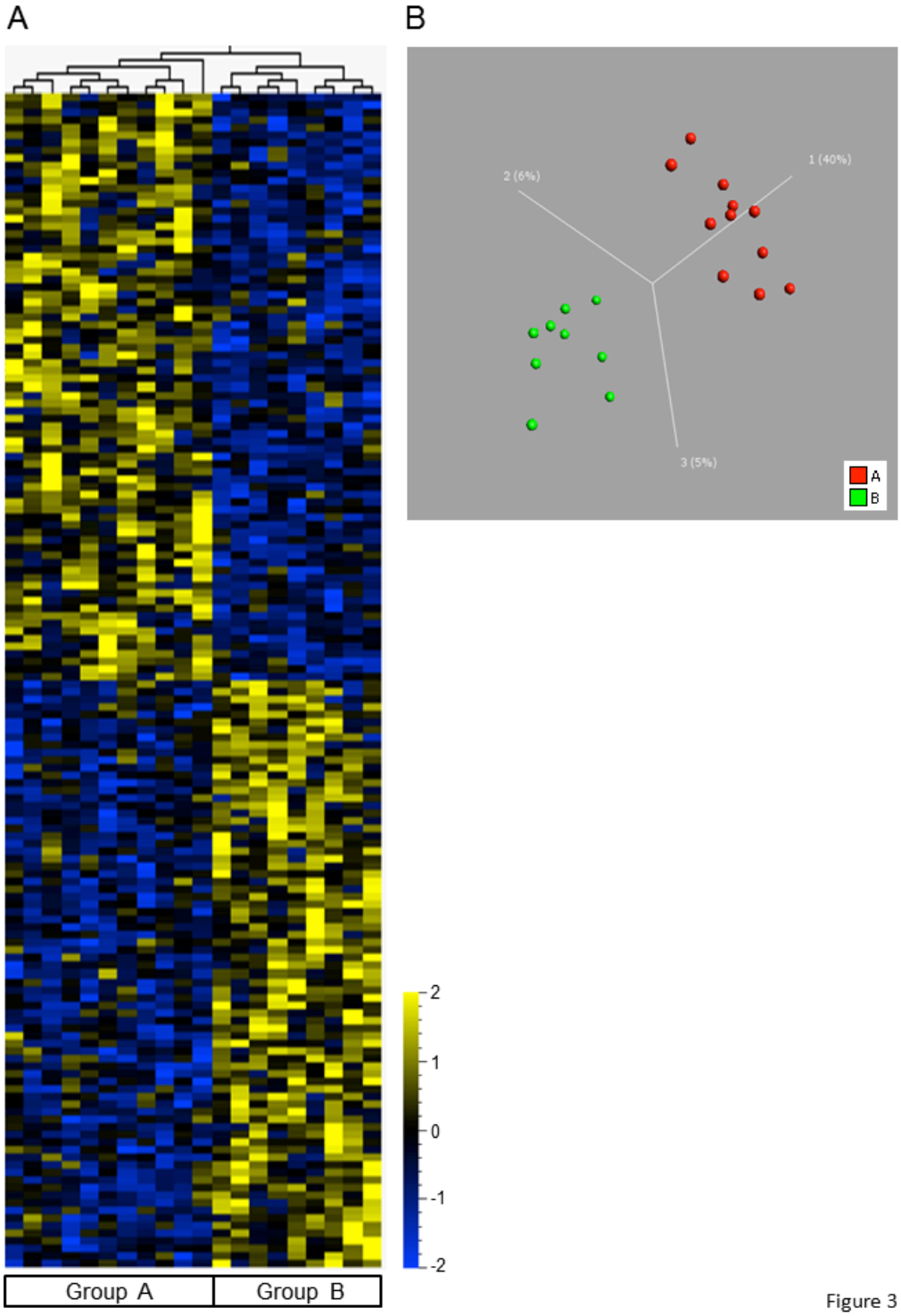


Figure 6.3 miRNA expression distinguishes slow and fast progressors of HCV post transplant. (A) Hierarchical cluster analysis and cladogram for Groups A and B (*P*<.01). (B) Principal Component Analysis for the same data-set.

3.3 miRNA expression is associated with slow progression of HCV post liver transplant

Of the total 152 miRNAs differentially expressed between Groups A and B, 76 miRNAs were down regulated and 76 were up-regulated in Group A relative to B. The data set of both down-regulated and up-regulated miRNAs were individually analysed using Metacore (GeneGo Inc.) to interrogate the genetic pathways regulated by these miRNAs. This algorithm recognises co-regulated components of pathways or biological processes that are impacted on by increased or decreased expression of specific miRNAs. Up regulation of a given miRNA results in increased inhibition (down regulation) of its target genes, whilst reduced miRNA expression reverses this inhibition leading to target gene up-regulation.

Analysis using Metacore of the up-regulated and down-regulated miRNAs between slow- progressors and fast- progressors, identified 7 networks (6 up-regulated and 1 down-regulated) of high statistical significance (Figure 6.4A). These networks regulate many pathways that are implicated in mediating HCV progression and are outlined below:

A

i

Network	P	Z	G
miRNA-200a, miRNA-141	2.39E-18	39	39
miRNA		**Target**	
↑miRNA-200a		↓TGFβR2	
↑miRNA-141		↓TGFβR2	

Network	P	Z	G
miRNA-203, miRNA-146a	2.33E-05	13	13
miRNA		**Target gene**	
↑miRNA-203		↓ SMAD4	
↑miRNA-146a		↓SMAD4	

Network	P	Z	G
miRNA-146a, miRNA-19a	2.67E-09	21	21
miRNA		**Target**	
↑miRNA-146a		↓EGFR	
↑miRNA-19a		↓EGFR	

ii

Network	P	Z	G
miRNA-20a, miRNA-20b, miRNA-205	2.02E-11	25	25
miRNA		**Target**	
↑miRNA-20b		↓ VEGFA	
↑miRNA-205		↓ VEGFA	
↑miRNA-20a		↓ VEGFA	

iii

Network	P	Z	G
miRNA-146a, miRNA-33a, miRNA-let7e	$1.96E^{-15}$	52	52
miRNA		**Target**	
↑miRNA-146a		↓IL-8/IL-6/CCL8/CD40L	
↑miRNA-33a		↓IRS2	
↑miRNA-let7e		↓TLR4	

iv

Network	P	Z	G
miRNA-34c-5p, miRNA-342-5p, miRNA-181c-5p, miRNA-29a, miRNA-19a, miRNA-150	1.84E-18	39	39
miRNA		**Target**	
↓ miRNA-150		↑c-Myb	

v

Network	P	Z	G
miRNA-205, miRNA-204, miRNA-20a, miRNA-146a, miRNA-328	1.25E-13	30	30
miRNA		**Target**	
↑miRNA-146a		↓ STAT1	
↑miRNA-20a		↓ TGFβR2	

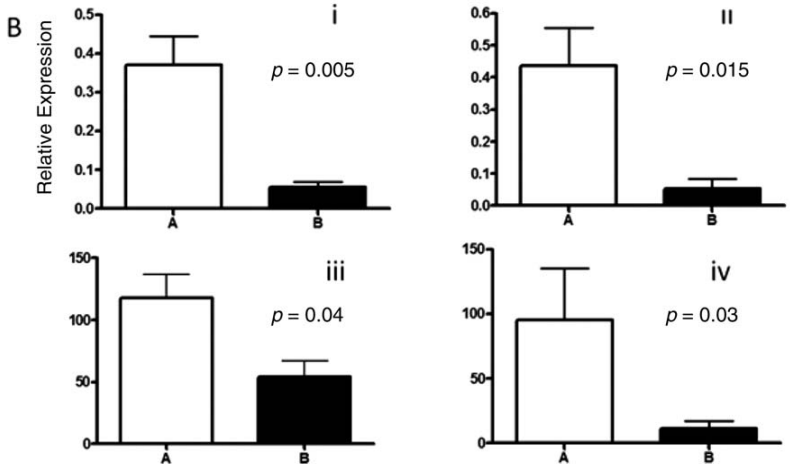


Figure 6.4. miRNA expression distinguishes slow HCV progressors (group A) from fast HCV progressors (group B) after transplantation. (A) A MetaCore analysis of group A versus group B ($P < 0.01$) identified statistically significant networks of miRNAs and known target genes implicated in (i) fibrogenic, (ii) angiogenic, (iii) inflammatory, (iv) apoptotic, and (v) mixed pathways. (B) A quantitative PCR analysis of a select group of miRNAs that were identified by a MetaCore analysis confirmed the up-regulation of (i) miRNA-19a, (ii) miRNA-20a, (iii) let-7e, and (iv) miRNA-146a expression in group A versus group B. Small nucleolar RNA SNORD66 was used as a reference RNA for normalization. Values are expressed as means and standard deviations and are representative of 3 different experiments. *P* values for each miRNA are indicated on the graphs.

3.3.1 Anti-fibrotic pathways (Figure 6.4A, i)

Three statistically significant networks of miRNA were identified that are associated with anti-fibrotic pathways: miRNA-200a and miRNA-141 (*P* value 2.39 x 10^{-18}), miRNA-203 and miRNA-146a (*P* value 2.33 x 10^{-5}) and miRNA-146a and miRNA-19a (P value 2.67x10^{-9}). Increased expression of both miRNA-203 and miRNA-146a are known to lead to down regulation of SMAD4. This acts as a tumour suppressor that functions in the regulation of the TGFβ signal transduction pathway, an important pro-fibrotic pathway. Increased miR-200a and miRNA-141 expression is known to mediate direct down regulation of TGFβR2. Increased expression of miRNA-146a and miRNA-19a leads to down-regulation of EGFR.

3.3.2 Anti-angiogenic pathways (Figure 6.4A ii)

One miRNA network that is known to inhibit angiogenesis was identified: Increased expression of miRNA-20a, miRNA-20b and miRNA-205 (*P* value 2.02x 10^{-11}) leads to down-regulation of VEGF-A activity.

3.3.3 Anti-inflammatory pathways (Figure 6.4A iii)

One network was associated with regulating the expression of genes promoting anti-inflammatory pathways (miRNA-146a, miRNA-33a, let-7e, *P* value 1.96x10^{-15}). Increased miRNA-146a expression is known to inhibit expression of pro-inflammatory mediators including IL-8, IL-6, CCL8 and CD40L. In addition, increased miRNA-33a expression leads to decreased IRS-2 activity and increased let-7e expression to decreased TLR4 expression.

3.3.4 Anti-apoptotic pathways (Figure 6.4A iv)

A single statistically significant network of down-regulated miRNAs was identified that regulates anti-apoptotic pathways (miRNA-19a and miRNA-150, *P* value $1.84x10^{-18}$). Decreased miRNA-150 expression causes up regulation of cMyb and decreased miRNA-19a expression leads to up-regulation of N-Myc expression.

3.3.5 Other pathways (Figure 6.4A v)

An additional network of differentially expressed miRNA that we identified (miRNA-205, miRNA-204, miRNA-20a, miRNA-146a, miRNA-328, *P* value $1.25x10^{-13}$) inhibits expression of genes known to promote HCV disease progression. Increased miRNA-146a leads to decreased STAT-1 expression and increased miRNA-20a to decreased expression in TGFβR2.

3.4 Validation and quantification of miRNA expression by PCR.

In order to independently validate the changes in miRNA expression we observed in Groups A and B and to quantify miRNA expression, we selected a subgroup of miRNAs from the GeneGo analysis outlined above (Figure 6.4A). Quantitative PCR analysis confirmed the up regulation of miRNA-19a, miRNA-20a, let-7e and miRNA-146a in Group A relative to Group B (Figure 6.4B). In addition, I validated miRNA expression changes detected in the microarray analysis by carrying out quantitative PCR for a random selection of miRNA (Table 6.2).

miRNA	Group A	Group B	P value
4304	29.69+/-27.2	24.91+/-16.6	0.69
1407-3	377.8 +/- 314	795.22 +/- 376	0.11
Let7f	17.17+/-15.14	12.04+/-10.25	0.5

Table 6.2 Random selection of miRNA expression changes using quantitative PCR

In order to investigate whether any of the miRNAs that I identified could represent tractable, non-invasive, and prognostic biomarkers for HCV recurrence, a quantitative PCR analysis was also performed on serum samples taken at a median of 6 months after transplantation (range 4-10 months) from fast and slow progressors. My results demonstrated specific and statistically significant up-regulation of miRNA-19a and miRNA-201 but not miRNA-150 or miRNA-146a in group A versus group B (Figure 6.5).

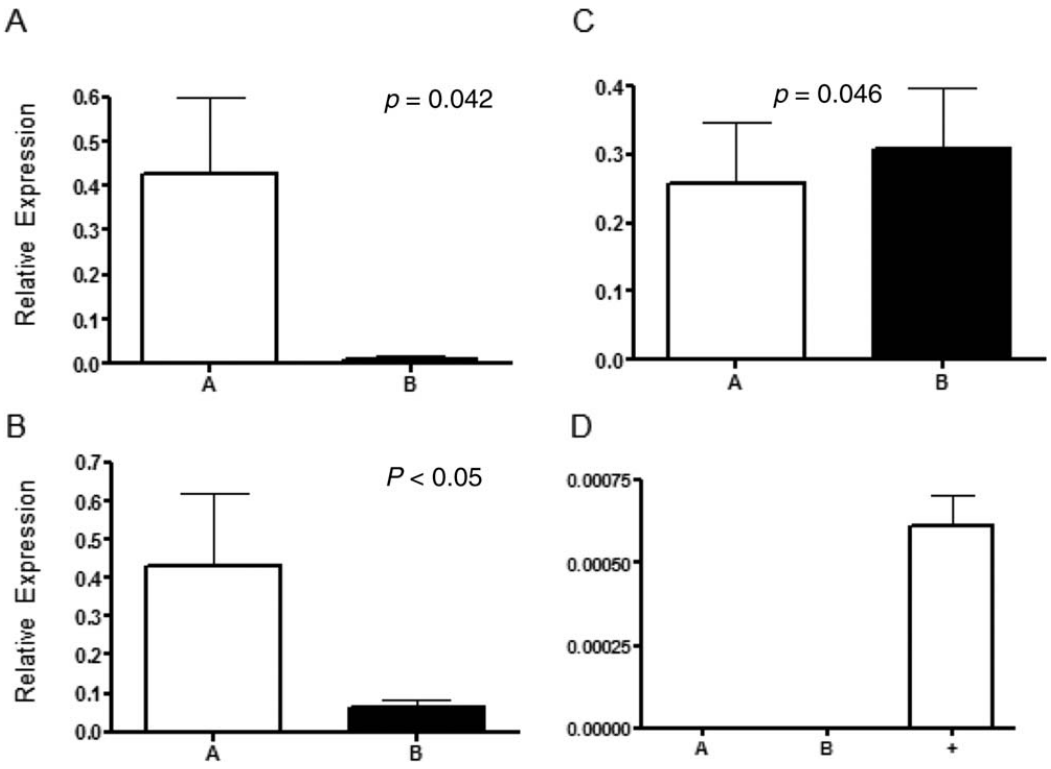


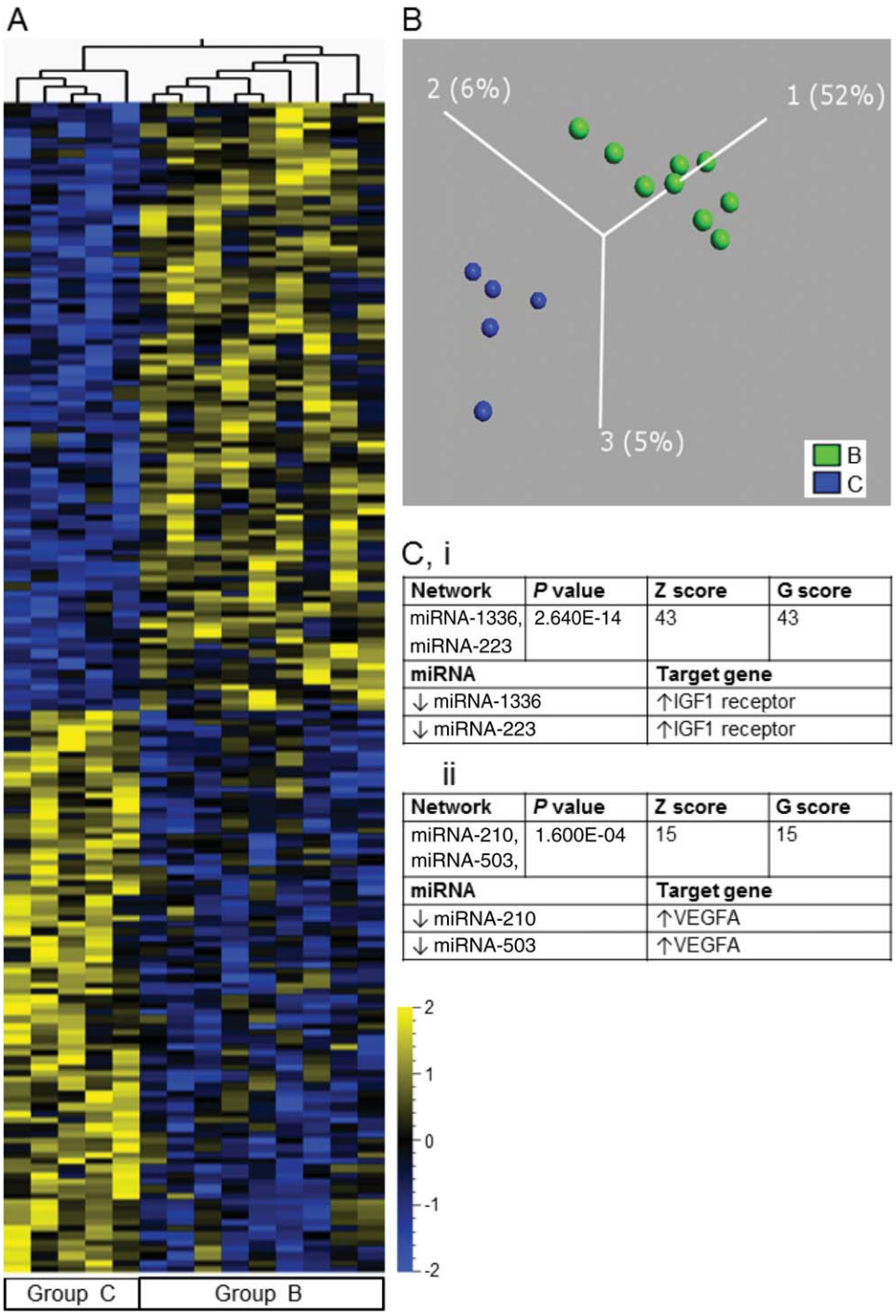
Figure 6.5 Serum miRNA expression distinguishes slow HCV progressors (group A) from fast HCV progressors (groupB) after transplantation. Serum samples were analysed by quantitative PCR to determine the expression of (A) miRNA-19a, (B) miRNA-20a, and (C) miRNA-150 in groups A and B. SNORD66 was used as a reference RNA for normalisation. Values expressed as means and standard deviations and representative of 3 different experiements. P values for each miRNA are indicated on the graphs. (+) represents RNA extracted from cells that are transfected with miRNA-146a.

3.5 miRNA expression distinguishes post-transplant HCV related fast- progressors from acute cellular rejection

Given the known difficulties in distinguishing post-transplant HCV recurrence from acute cellular rejection, we investigated miRNA expression in Groups B and C. I reasoned that differential miRNA expression between these groups may not only elucidate mechanistic insights but identify potential biomarkers that discriminate these clinical entities.

HCA analysis demonstrated distinct miRNA expression profiles for Groups B and C (Figure 6.3*A, P*<.01), with the cladogram above the heatmap (Figure 6.5*A*) as well the associated PCA (Figure 6.5*B*) confirming stronger similarity within each group than between groups. 190 miRNAs of statistical significance were differentially regulated between the two groups with 100 miRNAs down-regulated in Group B relative to Group C and 90 up-regulated (See Appendix II, Table 2).

Metacore analysis (GeneGo Inc.) revealed two highly statistically significant networks of down regulated miRNAs in Group B relative to Group C (Figure 6.3*C*). The first network consisted of miRNA-1336 and miRNA-223 (Figure 6.4*Ci, P* = 2.6×10^{-14}). Down regulation of both these miRNAs is associated with increased expression of IGF-1 receptor, which has been implicated in promoting liver fibrogenesis. The second network consisted of down regulation of miRNA-210 and miRNA-503 (Figure 5*Cii, P* = 1.6×10^{-4}) and is associated with pro-angiogenic pathways through the increased expression of VEGF-A.



C, i

Network	*P* value	Z score	G score
miRNA-1336, miRNA-223	2.640E-14	43	43
miRNA		**Target gene**	
↓ miRNA-1336		↑IGF1 receptor	
↓ miRNA-223		↑IGF1 receptor	

ii

Network	*P* value	Z score	G score
miRNA-210, miRNA-503,	1.600E-04	15	15
miRNA		**Target gene**	
↓ miRNA-210		↑VEGFA	
↓ miRNA-503		↑VEGFA	

Figure 6.6 miRNA expression distinguishes fast HCV progressors (group B) after transplantation from patients with ACR (group C). (A) HCA and cladogram fro groups B and C ($p<0.01$). (B) PCA for the same data set. (C) A MetaCore analysis identified 2 statistically significant networks of down regulated miRNAs and known target genes for individual miRNAs.

4.Discussion

In this study, I have demonstrated distinct intragraft miRNA gene expression between patients with slow fibrosis and patients with fast fibrosis after transplantation for HCV. Samples taken from Groups A and B were carefully selected and rigorously matched for known factors that strongly influence HCV recurrence post-transplant (i.e. donor age, cold ischaemia time, donor risk index, IL-28B recipient genotype and immunosuppression). Although this approach limited the sample size, it allowed me to identify changes in the expression of miRNAs of high statistical significance that are exclusively associated with HCV recurrence and fibrosis progression (Charlton et al., 2011, Berenguer et al., 2002). miRNA studies can sometimes be interpreted as a 'fishing' exercise with a host of miRNAs randomly selected. By vigorously and meticulously selecting the patient samples and matching them extensively for the known risk factors that influence HCV fibrosis progression post transplantation, I was able to identify specific miRNAs. Matching these cohorts was extremely time consuming but was essential to the design of this study and therefore is one of the key strengths.

The clinical significance of identifying patients with a fast fibrosis phenotype cannot be overstated. Recurrence of HCV post-transplant is the leading cause of graft loss and the commonest indication for consideration of re-transplantation (Berenguer, 2002). Although the main aim of this study was to identify potential mechanisms relating to HCV recurrence post-transplantation, using quantitative PCR from serum, I was also able to demonstrate that specific miRNAs (miRNA-19a and miRNA-20a) could

represent potential serum biomarkers for fibrosis progression. I also found that intra-graft miRNA expression in patients with evidence of HCV recurrence post LT differs from that in patients with acute cellular rejection and patients with normal liver biopsy findings after LT.

The miRNAs networks I have demonstrated to correlate with HCV disease progression identify molecular pathways of relevance to the pathogenesis of HCV recurrence. Although these pathways are identified through the regulation of known target gene expression, a MetaCore analysis revealed that these pathways have high statistical significance and, therefore, relevance. According to miRNA expression, profibrotic pathways would be down-regulated in group A versus group B. Key fibrogenic mediators including SMAD4 and TGFβR2 are known to be down-regulated in response to up-regulation of miRNA-203, miRNA-146a, miRNA-200a and miRNA-141 respectively. TGFβ is the main fibrogenic chemokine and is activated in response to liver injury, whereas SMAD proteins are intracellular mediators of signal transduction pathways of the TGFβ super-family members (Friedman, 2008b). Increased expression of miRNA-20a is known to lead to decreased expression of TGFβR2, which has been shown to attenuate the TGFβ signalling pathway and hepatic stellate cell (HSC) activation (Nakamura et al., 2000). Another important pathway in the pathogenesis of hepatic fibrosis is the deposition of the extracellular matrix (ECM), which originates from myo-fibroblastic cells derived from HSC and portal fibroblasts (Cassiman et al., 2002). The activation of EGFR on ECM-producing cells has been shown to contribute to the pro-fibrogenic phenotypic state (Bachem et al., 1989, Yang et al., 2003). EGFR gene

expression is inhibited by increased expression of miRNA-146a and miRNA-19a, as exhibited by the slow-progressor group.

Angiogenesis and inflammation are also known to contribute to hepatic fibrosis. New blood vessel formation, sinusoidal modelling and stellate cell expansion are integral and are mediated by mediators such as VEGF and PDGF (Lee et al., 2007). Up-regulation of the network consisting of miRNA-146a, miRNA-205 and miRNA-20a in the slow-progressor phenotype would be expected to inhibit VEGFA expression. Furthermore, increased expression of miRNA-146a is known to negatively regulate expression of IL-8: high levels of IL-8 (CXCL8) are associated with disease progression and poor response to interferon based treatment (Polyak et al., 2001b). The expression of pro-inflammatory mediators IL-6, chemokine ligand 8 (CCL8) and CD40L are also known to be inhibited by these changes in miRNA expression. TLR4 is the receptor for bacterial lipopolysaccharide on Kupffer cells and is also expressed on HSC. It is essential in the inflammatory response and plays an important role in the fibrogenic response. Specific single-nulceotide polymorphisms of TLR4 have been shown to contribute to the rate of fibrosis in HCV infection (Huang et al., 2007). My data also showed increased expression of let-7e, a significant inhibitor of TLR4 expression.

c-Myb, the prototype oncogene, plays an essential role in the regulation of cell development and differentiation (Oh and Reddy, 1999). An elevation of miRNA-150 is known to result in decreased expression of c-Myb and thus in cell apoptosis (Ladu et al., 2008, Malaterre et al., 2007). My finding of decreased miRNA-150 indicated

increased c-Myb expression and reduced apoptosis, therefore, a reduced rate of fibrosis in group A.

My results identified an important role for miRNA-146a in the pathogenesis of HCV recurrence after LT. miRNA-146a is up-regulated in response to TLR4 stimulation in monocytes and controls endotoxin tolerance so that the innate immune response favours cell survival (Taganov et al., 2006). Murine models also suggest that miRNA-146a is critical for regulatory T cell function and acts a 'molecular brake' for inflammation, myeloid cell proliferation and oncogenic transformation (Boldin et al., 2011, Lu et al., 2010). Although intragraft miRNA-146a expression was increased in the slow fibrosis progression group, there was no comparable increase in miRNA-146a levels in the serum. This phenomenon of increased expression of miRNAs in tissue but not serum or plasma is well described and arises due to the fact that only specific miRNAs are released by the secretion of microsomes including exosomes, and therefore only specific miRNAs are selectively packaged into microvesicles (Kosaka et al., 2010b, Zhang et al., 2010). I, therefore hypothesise that miRNA-146a plays an integral role in determining the rate of HCV recurrence post-transplant but is not a suitable serum biomarker candidate. A possible therapeutic intervention could be to increase miRNA-146a which would result in a slower, less aggressive fibrosis progression in HCV patients following liver transplantation.

My results identify a panel of miRNAs that not only determine whether a patient has a fast or slow fibrosis phenotype but could potentially also identify patients who require

treatment of their HCV earlier. Assessment of these miRNAs once steady liver graft function is achieved could lead to early consideration of anti-viral therapy.

Histology remains the gold standard for differentiating ACR from recurrent HCV infection, although it remains non-specific and subject to inter and intra-observer bias (Regev et al., 2004). HCV recurrence typically manifests as a lobular hepatitis with hepatocyte changes including ballooning, acidophilic body formation and steatosis. ACR is typically characterised by predominant portal changes consisting of a mixed portal inflammatory cell infiltrate, bile duct damage and portal vein endotheliitis (Snover et al., 1984). The two conditions may coexist, or even trigger each other, and their histological manifestations may also overlap. It is a common clinical scenario which, if diagnosed incorrectly, can have potentially deleterious consequences, such as the inappropriate administration of corticosteroids for presumed ACR, resulting in the potentiation of HCV viraemia. A limitation of the my study is that analysed biopsy samples from individuals with histologically diagnosed ACR were obtained at the time of suspected acute rejection and these were compared to protocol liver biopsy samples at 1 year from individuals with rapid fibrosis. It is therefore unclear as to whether the observed changes in miRNA expression predict the development of a specific injury phenotype or occur as a result of an already established pattern of injury within the allograft. A recent study demonstrated an increase in miRNA-122 and miRNA-148a in parallel with serum aspartate aminotransferase and alanine aminotransferase levels in patients with ACR (Farid et al., 2012). Comparing groups B and C, I was able to identify pro-fibrogenic miRNAs, through regulation of IGF-1 receptor and VEGF-A related pathways, that could help differentiate between rHCV

and ACR. The relatively small numbers in group C, however, make for statistically valid but cautious conclusions and require validation in larger prospective cohorts. To further investigate my findings, further comparisons of serial samples taken from individuals during similar phases after liver transplantation are required. This should also include early sampling before liver injury is established.

From a review of the current literature, it is clear that miRNAs are viewed as attractive, potential biomarkers because of their stable, cell-free form in blood. Critically, what remains unclear is how miRNAs make their way into the bloodstream: whether it is the result of cell death or secondary to active secretion from tissue cells? There is increasing evidence that serum/plasma concentrations of miRNAs are altered in multiple disease pathologies, including HCC (Kosaka et al., 2010a, Xu et al., 2011, Fan et al., 2008). Studies specific to HCV demonstrate that miRNA-122 expression correlates with serum transaminases and necro-inflammatory activity on liver biopsy, but not with fibrosis stage or parameters of liver function in patients with chronic HCV (Bihrer et al., 2011). miRNA-122 levels have also been shown to be decreased in patients who do not respond to anti-viral therapy compared to responders in the non-transplant setting (Sarasin-Filipowicz et al., 2009). My analysis did not find miRNA-122 to be expressed differentially between Group A and B, and this is not surprising because these groups were selected exclusively by differential fibrosis rates. Quantitative PCR performed on serum identified miRNA-19a and miRNA-20a to be increased in group A versus group B. Because of their potential mechanistic role in the pathogenesis of HCV recurrence, their detection in serum indicates that they may represent candidate biomarkers that can delineate fibrosis rates associated with HCV

recurrence after transplant. miRNA-20a has been studied in HCV infected patients pre-transplantation and healthy controls (Shrivastava et al., 2013). Serum miRNA-20a correlated with HCV fibrosis stage especially in the setting of chronic infection further highlighting its suitability as a biomarker for HCV mediated liver disease progression.

In addition to providing diagnostic information, miRNAs provide the possibility of therapeutic intervention. Novel targeted anti-miRNA therapies, so-called antagamirs, have been postulated as future therapeutic targets. In two separate murine models, the introduction of an anti-sense oligonucleotide/antagamir resulted in a reduction of hepatic steatosis and tumorgenesis repression, respectively (Kota et al., 2009). Although the use of antagamirs remains in its infancy, recently published data using miravirsen, a potent inhibitor of miRNA-122 function, has demonstrated a dose-dependent reduction in HCV RNA titres (Janssen et al., 2013). To advance this field further, extensive miRNA profiling of both diseased and healthy tissue is required. Although I cannot definitively prove that loss of miRNA-146a is a cause or a consequence of HCV, a targeted antagamir or related therapeutic agent against HCV-related fibrosis and recurrence after LT would represent an exciting advance.

miRNAs appear to have a complex role, more than just the simple inhibition of target gene expression. Some questions remain unanswered including why and how some miRNAs enter the systemic circulation, the association of miRNAs and exosomes and the half-life of miRNAs and impact on disease severity.

In conclusion, this study has demonstrated that the fibrosis rate associated with HCV recurrence post-LT is associated with changes in networks of intragraft miRNA expression that can regulate pro-inflammatory, pro-angiogenic and pro-fibrogenic pathways. Meticulous patient selection has enabled us to identify key miRNAs that provide a unique insight into potential mechanisms involved in the recurrence of HCV after transplantation. My data suggests a pivotal role for microRNA-146a, in the pathogenesis of HCV recurrence after transplantation as it was identified in different mechanistic pathways. Using liver tissue and, more relevantly, serum, I identified a panel of miRNAs associated with the severity of HCV recurrence that could be used as a potential biomarker. Larger translational studies are required to investigate both the role of miRNAs in the pathogenesis of HCV recurrence and to validate tractable biomarkers that are predictive of aggressive recurrence after LT. Although the advent of the new DAAs will undoubtedly transform the therapeutic landscape, the early identification of patients with rapid fibrosis rates and the ability to distinguish viral recurrence from ACR, remain pivotal clinical endpoints for selecting suitable patients in the challenging post-LT setting.

Chapter VII

General Discussion

The year 2014 marks the 25th anniversary of the identification of HCV. Globally, HCV is a major health burden and chronic HCV infection remains one of the leading indications for liver transplantation worldwide. Following liver transplantation outcomes amongst HCV positive patients is inferior compared to other aetiologies. Recurrence of HCV and the ensuing accelerated fibrosis progression leads to inferior patient and graft survival. Historically, the 'Achilles heel' remained the inability to offer efficacious and tolerable therapies in the post transplant period which could help attenuate fibrosis progression. Naturally, attention has turned to the development and identification of predictors of rapid fibrosis progression.

When I started my thesis in 2009, the landscape for the treatment of HCV was starting to change. It was soon becoming obvious that pegylated interferon and ribavirin (PEG/RBV), so-long the stalwart, would not be the only therapeutic option available to patients. In 2011, the first generation protease inhibitors were introduced in combination with PEG/RBV in patients with genotype 1 disease. Although SVR rates improved in patients with minimal fibrosis and compensated disease, the applicability in the post-transplant setting was anticipated to be difficult due to the increased side-effect profile, long treatment durations and potential drug-drug interactions with immunosuppression. Since then, the exponential increase in the development of new directly DAAs with and without PEG in the post-transplant setting has raised hopes of improving outcomes for this patient cohort. The new DAAs have undoubtedly impacted on the relevance of my thesis and its applicability to patients.

There has undoubtedly been a paradigm shift in the management of HCV recurrence post liver transplantation. Rather than wait for evidence of histological recurrence post transplantation to instigate anti-viral therapy, treatment is now being considered pre-liver transplantation to help reduce re-infection post transplantation. PEG can precipitate hepatic decompensation in patients with cirrhosis leading to significant morbidity and mortality. Treating patients active on the liver transplant waiting list has therefore been a contentious issue. However, data using the new DAAs would suggest that that we can certainly increase the number of patients entering liver transplantation with an undetectable HCV viral load, thereby reducing the number of patients developing HCV recurrence and rapid fibrosis progression (Curry MP, 2013). The new DAAs also heralds the dawn of potential salvage therapy and fibrosis regression. This approach may mirror the experience and results in chronic HBV and the use of the oral nucleos(t)ide agents. Recent data taken from a multi-centre European study demonstrated a cumulative incidence of inactivation and delisting for liver transplantation was 16% and 0% at 24 weeks, 27% and 10% at 48 weeks and 33% and 19% at 60 weeks following DAA treatment (Belli et al., 2016). 34 patients from a total of 103 were successfully inactivated, all of whom demonstrated an improvement in MELD and CPS. Baseline MELD, delta MELD and delta albumin (both assessed 12 weeks post DAA therapy) were identified as predictors of inactivation from liver transplantation. These data would suggest that the 'Holy Grail' of fibrosis regression and clinical improvement with the use of DAAs is not as impossible as first thought. Longer term benefits of DAA therapy however is still required. It should also be highlighted that DAA therapy comes with a financial burden, one which certain Nations may not be able to afford.

Numerous factors influencing HCV recurrence post liver transplantation have been established. However, the group of patients that need to be identified early are those with rapid fibrosis progression. The definition of rapid fibrosis progression I used in this thesis, which is well validated, is the development of F≥2 at 12 months (Firpi et al., 2004). The development of F≥2 has added importance as it also an indication for consideration of anti-viral therapy. In the three sets of experiments I performed, patients were catergorised as having slow fibrosis progression (F<2) or fast fibrosis progression (F≥2).

Chapter IV addressed the role of donor and recipient rs12979860 IL-28B polymorphisms in HCV recurrence post liver transplantation. My results were in keeping with other published series from North America, Europe and Asia. Although my data remains unpublished, it appears to be the only UK based study evaluating the role of donor and recipient IL-28B genotype in the post transplant period. Recipient non-CC rs12979860 IL-28B genotype were more likely to develop F≥2 at 12 months and progress to F≥4 post transplantation. In addition, patients with donor/recipient rs12979860 IL-28B CC combination were less likely to develop F≥2 at 12 months and F≥4. The donor/recipient rs129779860 IL-28B CC combination was associated with a 100% SVR rate but only 6 patients were included. The findings from this experiment were not new or unique to the scientific world. However, it served a purpose both for my Institution in defining the frequency of the rs12979860 IL-28B genotype amongst its patient cohort and for me to develop the remaining sets of experiments.

When I started investigating the role of CXCL10 in predicting HCV recurrence post transplantation there was limited data available. Given that CXCL10 is produced by HSCs and is also a marker of ISG expression, it made it an extremely attractive serum marker for fibrosis progression and a marker to help predict response to AVT respectively. CXCL10 has been investigated in patients with HCV mono-infection and in HIV/HCV co-infected patients undergoing AVT pre-transplantation. Three studies, including my own have established that CXCL10 is a robust predictor of fibrosis progression post liver transplantation in patients with HCV recurrence (Berres et al., 2011). The combination of recipient rs12979860 IL-28B non-CC genotype and CXCL10 levels pre-transplant was an interesting finding which helped improve the identification of patients that were likely to respond to AVT. When submitting my paper for publication some of the comments I received related to the relevance of CXCL10 in the era of potentially interferon-free regimens. My rebuttal to the reviewers and my stance is that although these new regimens are on the horizon, at the time of performing my experiments, no such therapies were available outside the remits of a clinical trial. The majority of patients I studied treated with AVT were treated due to clinical necessity. Waiting for newer therapies was not an option and they were certainly not suitable candidates for a clinical trial. A longitudinal assessment of CXCL10 expression pre and post transplant is an interesting future study.

CXCL10 clearly plays an integral role in host immune response to HCV infection. Its role post liver transplant still requires further investigation. Proposed further experiments should include the measurement of all forms of CXCL10 (total, long and short) to determine the correlation with treatment response and fibrosis progression.

Correlation with DPP4 levels is also required. Assessment of HCV specific IFN-γ producing T cells and CD56 dim and bright NK cells would also help improve the understanding of mechanisms for HCV persistence and accelerated frbrosis progression post transplantation. The expression of CXCR3 on NK and T cell populations should also be undertaken. These proposed experiments will now of course be conducted in the DAA era. Data in patients pre-liver transplantation with the combination of sofosbuvir and ribavirin has demonstrated DPP4 and CXCL10 were not viable biomarkers in predicting treatment outcome(Meissner et al., 2015).

My own study group has continued some of this work and studied 32 patients with compensated Child Pugh B or C cirrhosis that underwent 12 weeks of treatment with sofosbuvir with an NS5A inhibitor and ribavirin (Childs, 2015). CXCL10 levels were higher at baseline and TW12 in patients that did not achieve a SVR and declined during therapy. In patients that achieved a SVR there was an initial rise in CXCL10 levels from baseline to TW4. A cut off CXCL10 of < 250 pg/ml demonstrated a positive predictive value of 92% fro SVR. DPP4 were significantly higher at TW12 in patients that achieved SVR with levels increasing from baseline to TW12. CXCL10 and DPP4 levels positively correlated at baseline but an inverse correlation was evident at TW12. In the same cohort, we also investigated NK cell activation (Nkp30 expression) and NKG2D expression on treatment outcomes ((Childs, 2016). The data demonstrated higher Nkp30 expression at baseline, on treatment and at the end of treatment in patients that did not achieve a SVR suggesting increased NK activation and upregulated ISG expression.

The most exciting sets of experiments I performed were related to my miRNA work. It is clear to see that miRNAs offer an attractive insight into disease mechanisms. In addition, their stable cell-free form within blood and other tissues offers the opportunity to develop biomarkers. Finally, miRNAs provide the possibility of therapeutic intervention, so called antagamirs. Recently published data on miravirsen, a potent inhibitor of miRNA-122 function, demonstrated a reduction in HCV RNA (Janssen et al., 2013). Our understanding the role and application of miRNAs continues to grow and at present my study is only one of two studies performed using miRNAs to help predict HCV recurrence post liver transplantation (Gehrau et al., 2013). Numerous studies have now been performed using miRNAs to help predict disease recurrence, and acute cellular rejection following liver transplantation (Morita et al., 2016, Liese et al., 2016, Shaked et al., 2016)

When designing the miRNA experiments, I wanted to delineate the integral pathways involved in patients that develop rapid fibrosis progression by 12 months. These patients were then compared to a group of patients with slow fibrosis progression (F<2 at 12 months) that were matched for known factors that influence HCV recurrence i.e. CIT, donor age, HCV VL, recipient age etc. In essence, by removing these variables which are known to influence fibrosis progression, only true mechanistic pathways would be delineated. A control group of non-HCV patients with no fibrosis biopsied in the post-transplant period (group D) was important to demonstrate that any findings were unique to patients with HCV only. Finally, group C provided information into a common and sometimes very difficult clinical scenario, recurrent HCV versus ACR.

Differentiating between acute cellular rejection and recurrent HCV is an important decision to make which if made incorrectly can affect the natural course of the patient and graft. This is one of the key strengths of my miRNA experiments. The miRNA identified were specific to the cohorts studied and not due to randomly elected miRNAs. One of the criticisms of this paper when sent for peer review were the small numbers included in the final analysis. The numbers were limited due to the extensive matching of the known risk factors that are known to impact on HCV related fibrosis progression post liver transplantation.

My results demonstrated an integral role of miRNA-146a in the pathogenesis of HCV recurrence post transplantation and a potential role of miRNA-19a and miRNA-20a as potential serum biomarkers that can delineate fibrosis rates in this patient cohort. These data undoubtedly require further validation in prospective cohorts. We initially collected plasma from all patients undergoing liver transplantation for HCV (2011 onwards) to have a prospective cohort to potentially validate the role of miRNA-19a and miRNA-20a as fibrosis biomarkers. However, the advent of DAA changed our approach to patients with more being successfully cured with pre-transplant or following transplantation. Certainly, the need for robust predictors of rapid fibrosis progression in patients with HCV infection following liver transplantation has declined significantly given the highly efficacious AVT now available.

A cautious observation was made regarding differentiating between ACR and HCV recurrence given the small patient sample numbers involved. Further validation is certainly required here. The microRNA experiments I designed and performed have

formed the basis of further studies assessing fibrosis progression in other cohorts. In particular, we have used miRNAs to help develop biomarkers for progressive fibrosis in young adults following liver transplantation.

This thesis and the experiments I have undertaken exposed me to variety of scientific techniques ranging from PCR to microRNA gene expression. I believe it has therefore given an excellent grounding and platform to continue my scientific career. Although, my research interests are no longer based in HCV, a lot these techniques and in particular my understanding of miRNAs and their applications as biomarkers are very relevant to my new areas of interest. I am very grateful for all staff within the Institute of Liver Studies and King's College London who helped me with the design and execution of my experiments. I am also indebted and grateful to my patients without whom none of these experiments would ne possible.

In conclusion, the results in this thesis have shown that donor and recipient rs12979860 IL-28B polymorphisms (Chapter VI) and CXCL10 levels (Chapter V) can identify patients with rapid HCV recurrence post transplantation. CXCL10 levels pre-AVT were also a sensitive predictor of treatment response. Additionally, this thesis has also identified miRNAs of interest which are involved in the pathogenesis of HCV recurrence and may act as possible biomarkers of disease progression or therapeutic targets. The promise of the new DAAs appears to hold true and importantly, if made available to those in greatest need, then potentially, in 20 years time HCV infection should be a disease of our past.

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Appendix I

Table 1. List of miRNAs and associated P values for A V B analysis (P<0.01)

miRNA ID	p-Value
mir-2326	0.001172
mir-563	0.009708
mir-iab-4	0.001224
mir-223	0.003762
miR-194	0.00456
miR-103	0.005534
ENSG00000238520	0.005063
ENSG00000239111	0.004166
mir-10b	0.002498
ENSG00000202216	0.005209
mir-314	0.000867
miR-137	0.000168
MIR1861b	0.005573
mir-564	0.007159
mir-190	0.001304
mir-2837	0.002749
mir-183	0.000417
miR1043-3p	0.003758
mir-3136	0.002154
mir-124	0.006973
mir-1422e	0.000762
mir-197	0.000864
mir-3237	0.001014
mir-4054	0.002215
mir-3165	0.000169
mir-3400	0.008253
ENSG00000252525	0.003692
mir-193a	0.007815
mir-2162	0.000421
mir-3232	0.002789
mir-1994a	0.002895
miR-79	0.006883
miR-17-3p	0.007683
miR171c	1.39E-05
mir-150	0.009438
mir-519b	0.001113
mir-18	0.00905
miR-1421k-3-star	0.001283
mir-29a	0.0081
mir-1898	0.007452
mir-600	0.009785

miR-M23-2-star	0.006422
mir-22	0.009841
miR-137	0.009071
MIR408	0.00858
ACA22	0.001702
ACA67B	0.004362
ENSG00000252083	0.009607
mir-297c	0.001083
ENSG00000238670	0.008772
mir-30e	0.003905
mir-19a	0.001583
SNORD119	0.004023
ENSG00000238368	0.003267
ENSG00000238771	0.003048
mir-57	0.004803
miR-430a	0.002791
mir-4073	0.007559
MIR399l	0.008364
let-7g	0.006098
miR1037	0.006542
mir-34	0.000536
mir-4026	0.003873
ENSG00000212187	0.000603
ENSG00000251918	0.002678
MIR2638b	0.004389
mir-71	0.002298
MIR1882g	0.009824
miR-17-5p	0.006981
mir-2317	0.009262
mir-713	0.005657
mir-363	0.002742
MIR171e	0.003406
miR395b	0.00776
18-Oct-10	0.005815
mir-4140	0.007191
Affymetrix Control Sequence	0.003137
ENSG00000222687	0.009075
miR-2a	0.003692
mir-4309	0.006839
MIR807c	0.00568
mir-223	0.006502
miR-147	0.004749
mir-71	0.008359
miR-4102-3p	0.005088

mir-362	0.008745
miR-15a	0.007883
ENSG00000252295	0.002028
miR-306	0.006638
mir-126b	0.006358
mir-786	0.008306
mir-2472	0.006182
mir-264	0.007604
MIR156a	0.009183
miR396a	0.00593
miR-516a-3p	0.004657
MIR1863	0.000208
MIR1507	0.005189
miR-14	0.009346
mir-30a	0.007463
MIR399i	0.004987
MIR167c	0.007689
mir-1955	0.006448
mir-196b	0.008844
MIR1124	0.007816
mir-2496	0.008198
mir-2702	0.005983
mir-4262	0.002888
mir-144	0.00444
mir-3283	0.005838
mir-1335	0.007948
mir-33a	0.005656
MIR164e	0.004279
mir-669m-2	0.004131
MIR164c	0.005784
mir-690	0.000747
mir-146a	0.003909
mir-597	0.005136
mir-4304	0.009688
mir-669l	0.005936
mir-804	0.006915
MIR439h	0.007605
mir-153a	0.001942
miR167d	0.009573
mir-2067	0.006354
MIR159c	0.008715
let-7e	0.000985
ENSG00000252356	0.002627
mir-1356	0.002538

mir-434	0.00647
miR-199	0.003415
MIR399i	0.001988
mir-10d	0.004296
mir-551a	0.007877
miR2080	0.00291
ENSG00000221376	0.000424
mir-19a	0.003246
mir-310	0.008511
mir-600	0.001927
miR-20	0.000803
MIR159a	0.004897
mir-190	0.009234
mir-328	0.006409
miR1071-3p	0.009701
mir-455	0.000487
mir-4181	0.006636
MIR473b	0.004417
miR-210-star	0.00655
mir-307	0.005373
miR529b	0.006898
mir-3596	0.001978
mir-1470	0.004883
let-7f-1-star	0.005631

Table 2. List of miRNAs and associated P values for B v C (P<0.01)

* Includes cross species miRNA

miRNA ID	p-Value
mir-320a	0.00817046
mir-289*	0.00236549
mir-320	0.00989673
mir-289*	0.00851291
mir-289*	0.0076023
mir-289*	0.00717525
mir-2392	0.002981
miR-689	0.00568342
mir-455	0.00549904
mir-1910	0.00525964
MIR166d	0.00067784
miR166d	0.00313047
MIR394a	0.00667694
mir-1263	0.00187591
mir-940	0.0055436
mir-202	0.00611854
mir-628	0.00750827
SNORD123	0.00602116
mir-223	0.00729448
ACA61	0.00829265
miR166b	0.00359462
mir-210	0.00397882
mir-2277	0.00821367
mir-2323	0.00264089
MIR1846e	0.00491657
U80	0.00720783
ENSG00000239188	0.00464036
mir-378	0.00111964
miR-220c	0.00756824
mir-3428	0.00159052
MIR854a	0.00932175
mir-2413	0.00876128
U82	0.00996965
mir-503	0.00723453
mir-9-3	0.00846339
mir-671	0.00763588
mir-1401	6.1108E-05
mir-1643	0.00688866
miR-365	0.00198826
miR-4315	0.00299569
mir-237	0.00733895
mir-85	0.00174727
Bantam	0.00236806
18-Oct-10	0.00547017
mir-1464	0.00924842

mir-337	0.00964521
miR395j-star	0.00658693
mir-2368	0.00105112
mir-1410	0.00116467
mir-133	0.00187636
ENSG00000238657	0.00070159
ENSG00000238438	0.00140048
mir-148a	0.00451605
MIR319d	0.00918419
mir-337	0.00662734
mir-4183	0.00589984
mir-26a-2	0.00063753
mir-210	0.00289675
mir-67	0.00394548
mir-181b	0.00223169
ENSG00000252727	0.00382186
mir-148a	0.00843693
mir-4293	0.0086899
mir-988	0.00939586
ENSG00000202482	0.00320417
mir-3183	0.00033484
MIR919	0.00598407
mir-139	0.00839438
miR444d.1	0.00723025
mir-143	0.00350321
mir-363	0.00381422
mir-760	0.00564874
miR1107	0.00764288
mir-93	0.00668002
ENSG00000238769	0.00507379
miR-196	0.00867026
mir-1670	0.00793923
mir-1199	0.00679438
miR-344-5p	0.00337892
mir-125b-2	0.00315251
mir-1224	0.00551367
mir-466j	0.00983431
mir-3274	0.00140485
miR-376a	0.00441709
mir-M4 // mir-M4	0.00617229
mir-3409	0.00379679
mir-276b	0.00729543
MIR2864	0.00653111
MIR2612b	0.00601176
mir-235	0.00961664
MIR172a	0.0035177
miR-281	0.0018786
U38B	0.00139754
miR-929	0.00583614
mir-28*	0.0042766

mir-217	0.00192598
18-Oct-10	0.0045974
mir-1335	0.00778665
MIR171b	0.00445857
mir-548d	0.00862925
MIR2652k	0.00919739
mir-15b	0.00322253
MIR393b	0.00718647
ENSG00000212278	0.00228669
mir-2139	0.00502854
mir-2768	0.00649514
MIR812c	0.00477941
mir-9-2	0.00636616
mir-994	0.0025873
mir-3165	0.00330041
ENSG00000200536	0.00671252
mir-654	0.00139629
mir-317	0.00431287
miR-2014	0.00787429
MIR167b	0.00828175
mir-872	0.00605112
mir-3168	0.00583524
MIR169m	0.00066973
miR1088-5p	0.00991275
mir-30a	0.00361269
mir-BART18	0.00200979
mir-745b	0.00911752
MIR164b	0.00803147
mir-2162	0.0016493
mir-1245	0.00903151
ACA2b	0.00975822
mir-278	0.00501571
MIR399j // MIR399j	0.00708858
miR-4006a-5p	0.00500232
mir-31b	0.00985076
MIR396b	0.00502022
mir-148a	0.0040028
MIR395l	0.00497851
mir-86	0.00327391
mir-2213	0.00851612
mir-2416	0.00535131
mir-2200	0.0076391
mir-148b	0.00036336
mir-28*	0.00299225
mir-331	0.00891322
ENSG00000206776	0.00645239
MIR1849	0.00912326
HBII-438B	0.00844318
miR-H4-star	0.00786807
v49_ENSG00000212347	0.00100756

mir-3179-1	0.00088137
MIR2907d	0.00144374
miR1079-3p	0.00417513
mir-1299	0.00332336
miR-430a	0.00299738
mir-15c	0.00861935
mir-74b	0.00484656
mir-230	0.00581304
mir-129-2	0.00649397
mir-520g	0.00137256
mir-34c	0.00049251
miR-1419b	0.00654366
miR-101-star	0.001209
mir-154	0.00198151
mir-2770	0.00264573
mir-137	0.00126422
miR-7-star	0.00632391
v49_ENSG00000212434	0.00451647
mir-304	0.00646883
ENSG00000206761	0.0028616
18-Oct-10	0.00207621
mir-3192	0.00094216
miR-137	0.00487657
mir-215	0.00923191
mir-3176	0.00020151
ENSG00000221611	0.00668056
mir-33	0.00119911
MIR2124i	0.00427338
miR-9	0.00062048
mir-3136	0.00481911
mir-24-2	0.00635902
mir-iab-4	0.00197567
ENSG00000239146	0.00738688
mir-315	0.00073754
mir-1289-1	0.00837568
mir-220c	0.00235516
18-Oct-10	0.00131801
mir-1298	0.00104294
mir-100	0.00110576
ENSG00000252669	0.00931313
mir-10b	0.0084654
MIR396c	0.00215018

Appendix II

Publications

Recurrent HCV infection post liver transplantation: mechanisms, assessment and treatment.
D Joshi, M Pinzani, I Carey, K Agarwal.
Nature Reviews Gastroenterology and Hepatology, 2014, Epub ahead of print.

CXCL10 levels identify individuals with rapid fibrosis at 12 months post-transplant for HCV and predict treatment response.
D Joshi, Carey I, Foxton M et al.
Clinical Transplantation 2014 May 28(5):569-78.

The treatment of genotype 1 chronic hepatitis C virus infection in liver transplant candidates and recipients.
D Joshi, I Carey, K Agarwal.
AP&T. 2013 Apr; 37(7):659-71

Distinct MicroRNA profiles are associated with severity of HCV recurrence and acute cellular rejection after liver transplant.
D Joshi, S Salehi, Brereton H et al.
Liver Transplantation. 2013 Apr; 19(4): 383-94

Appendix III

Oral presentations

The role of microRNAs in predicting fibrosis post liver transplantation for HCV.
Department of Hepatology and Gastroenterology Hannover Medical School, Hannover, Germany. April 2014.

Transplantation for viral hepatitis – current practice and future directions.
BASL School of Hepatology. Birmingham, UK. Nov 2013.

Antiviral therapy in HIV co-infection and Liver transplant.
Frontiers in Hepatology, London, UK. Nov 2012.

IL28B haplotypes and IP-10 predict treatment response for recurrent HCV post transplant. Oral presentation, Digestive disease forum, Liverpool, UK. June 2012.

Grand Round presentation: HCV recurrence post transplant.
International Liver Congress, Barcelona, Spain. April 2012.

Poster presentations

IP10 levels identify individuals with rapid fibrosis at 12 months post-transplant for HCV. D Joshi, I Carey, M Foxton, et al. A565, EASL 2013.

Distinct micro-RNA profiles are associated with severity of HCV recurrence and ACR after liver transplant. D Joshi, S salehi, H Brereton, et al. A691, AASLD 2012, Hepatology, Vol 56, No. 4.

IP-10 levels in HIV/HCV co-infection decrease with the initiation of successful AVT: implications for earlier ART in co-infected patients? K Childs, I Carey, M Bruce, et al. A979, AASLD 2012, Hepatology, Vol 56, No. 4.

Pre-treatment IP-10 concentrations are associated with a sustained virological response in HIV/HCV co-infected patients. D Joshi, M Bruce, A Suddle et al. Gut 2011;60:Suppl 2 A28. P60.